

## RESEARCH ARTICLE

# Developmental environment has lasting effects on amphibian post-metamorphic behavior and thermal physiology

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## ABSTRACT

Environmental challenges early in development can result in complex phenotypic trade-offs and long-term effects on individual physiology, performance and behavior, with implications for disease and predation risk. We examined the effects of simulated pond drying and elevated water temperatures on development, growth, thermal physiology and behavior in a North American amphibian, *Rana sphenocephala*. Tadpoles were raised in outdoor mesocosms under warming and drying regimes based on projected climatic conditions in 2070. We predicted that amphibians experiencing the rapid pond drying and elevated pond temperatures associated with climate change would accelerate development, be smaller at metamorphosis and demonstrate long-term differences in physiology and exploratory behavior post-metamorphosis. Although both drying and warming accelerated development and reduced survival to metamorphosis, only drying resulted in smaller animals at metamorphosis. Around 1 month post-metamorphosis, animals from the control treatment jumped relatively farther at high temperatures in jumping trials. In addition, across all treatments, frogs with shorter larval periods had lower critical thermal minima and maxima. We also found that developing under warming and drying resulted in a less exploratory behavioral phenotype, and that drying resulted in higher selected temperatures in a thermal gradient. Furthermore, behavior predicted thermal preference, with less exploratory animals selecting higher temperatures. Our results underscore the multifaceted effects of early developmental environments on behavioral and physiological phenotypes later in life. Thermal preference can influence disease risk through behavioral thermoregulation, and exploratory behavior may increase risk of predation or pathogen encounter. Thus, climatic stressors during development may mediate amphibian exposure and susceptibility to predators and pathogens into later life stages.

**KEY WORDS:** Frogs, Behavioral phenotype, Climate change, Critical temperatures, Developmental plasticity, Pond drying, Thermal physiology

## INTRODUCTION

For many organisms, development is a sensitive period during which environmental change can result in profound impacts on the adult phenotype (Monaghan, 2008; West-Eberhard, 2003). As anthropogenic change transforms climatic conditions, organisms will experience mounting environmental stressors during these critical windows, resulting in immediate and potentially long-term consequences for survival and fitness (Kirschman et al., 2018; McDermott Long et al., 2017). Although developmental plasticity can buffer the impacts of environmental variability on survival, plasticity often results in phenotypic trade-offs later in life (Brannelly et al., 2019; Kohli et al., 2019). Given the impact of climate change on biodiversity, it is critical to characterize how developmental plasticity in response to higher temperatures can shift the thermal tolerances of organisms into adulthood.

For organisms that experience very different environments across life stages, plastic responses to environmental conditions and resulting phenotypic changes during one stage may carry over to the next stage, with potentially negative consequences. The potential for mismatched responses and carry-over effects across developmental stages has important implications for population viability in the face of global change. For example, developmental acclimation to high temperatures increases critical thermal maxima in *Drosophila melanogaster*, with a larger and more long-lasting impact than adult acclimation (Kellermann et al., 2017). Conversely, in other organisms with complex life cycles, acclimation to thermal conditions experienced during one life stage (e.g. larval) may (Rebolledo et al., 2021; Ruthsatz et al., 2022, 2020) or may not carry over to the next life stage (Enriquez-Urzelai et al., 2019). Moreover, global change will result in not only higher average temperatures, but also increased temperature variability and more variable precipitation (Cook et al., 2018; Rahmstorf and Coumou, 2011; Vasseur et al., 2014), highlighting the need for characterizing developmental acclimation under natural conditions.

In addition to thermal acclimation, the developmental environment may lead to coordinated changes in other aspects of life history and behavior. When multiple traits are consistently expressed together across contexts, they are referred to as a syndrome (Sih et al., 2004). The pace-of-life syndrome (POLS) hypothesis predicts that life history traits, physiology and behavior fall along a slow-fast continuum that is driven by individual metabolic rate, in which ‘fast’ organisms have higher metabolic rates, faster growth, greater fecundity, and higher levels of activity, boldness and exploration (Careau and Garland, 2012). Recently, an extension to POLS was proposed, in which individuals consistently vary in their behavior and thermal preferences along a hot-cold continuum (Goulet et al., 2017a,b). Given the strong positive relationship between metabolism and temperature, this hypothesis predicts that organisms that consistently select higher temperatures and perform better at those temperatures are more active,

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exploratory and bold (Goulet et al., 2017b). This is particularly relevant for ectothermic organisms, because behavior and thermal physiology are inextricably linked via behavioral thermoregulation, which is required to achieve optimum body temperatures for performance (Abram et al., 2016; Angilletta et al., 2002; Briffa et al., 2013). Early life parameters, such as size at birth and growth rate, have been shown to influence both performance and behavioral phenotypes (Royauté and Dochtermann, 2017; Zablocki-Thomas et al., 2018). Furthermore, depending on the environment, behavioral phenotypes can impact predation risk and disease transmission (Kashon and Carlson, 2018; Kortet et al., 2010). If the early life environment can shift correlated traits via developmental plasticity, a warming world may have far-reaching consequences for survival not only during ontogeny, but also in adulthood.

Developmental plasticity is widespread in amphibians (Edge et al., 2016; Richter-Boix et al., 2011), making this taxon well suited to characterizing the role of developmental conditions on thermal physiology and behavior. Amphibians are also facing increasing changes in temperature and water availability throughout development (Kohli et al., 2019) and shifting pressures from pathogens post-metamorphosis (Cohen et al., 2019). In addition, there is evidence that individual thermal preferences and other aspects of amphibian thermal physiology may play a role in disease outcomes (Cohen et al., 2017; Sauer et al., 2018). Although thermal acclimation during development and post-metamorphosis has been investigated in some amphibian species (Enriquez-Urzelai et al., 2019; Seebacher and Grigaltchik, 2014), the effects of both environmental pond drying and warming on correlated traits have yet to be explored.

To test the carry-over effects of developmental stressors on amphibian physiology and behavior, we raised southern leopard frog (*Rana sphenoccephala*) tadpoles in outdoor mesocosms *in situ* under different drying and warming regimes. After metamorphosis, we tested the impact of rearing environment on thermal physiology by measuring critical thermal maxima and minima ( $CT_{max}$  and  $CT_{min}$ , respectively) and the thermal sensitivity of jumping performance. Then, we examined the direct and indirect impacts of the developmental environment on individual frog exploratory behavior and thermal preference and tested whether behavior is predictive of that selection. We predicted that larvae would accelerate development in response to drying and warming, subsequently resulting in reduced jumping performance across all temperatures. Furthermore, we predicted that a warmer developmental environment would result in a higher  $CT_{max}$  and  $CT_{min}$ , the selection of warmer temperatures, and increased exploratory behavior (e.g. more time spent in arena center, larger areas explored, more lines crossed) in open-field tests, based on POLS theory (Goulet et al., 2017b). This work characterizes long- and short-term effects of putative climatic stressors during development on amphibian behavior and thermal performance, which are inherently linked via behavioural thermoregulation. Our results have implications for understanding how a changing climate will impact amphibians sub-lethally via carry-over effects, potentially shifting population means in thermal physiology and behavior and impacting persistence in the face of global change.

## MATERIALS AND METHODS

### Animal rearing in mesocosms

We collected four *Rana sphenoccephala* (Cope 1889) egg masses laid within 1 week of each other in Fort Polk, Louisiana, USA (31.126991, -93.0142648), on 5 January 2018. Eggs were

transferred to 55 liter plastic tubs and allowed to develop outside in shallow pond water (~20 liters) from the egg collection location. To rear tadpoles, we prepared a 3×6 array of 18 cattle tanks (Rubbermaid, 1135 liters) in Simpson, Louisiana (31.254400, -93.057732), near the location of egg collection. Tanks were equipped with adjustable drainpipes to allow for water depth reduction in drying treatments and to ensure that the maximum water depth remained at 40 cm after large precipitation events.

Following the methods of Brannelly et al. (2019), we covered tanks with 50% shade cloth, filled them with 600 liters of residential water, and allowed the water to age 48 h. We then added locally sourced dried leaves (200 g, mix of hardwoods and pine) and rabbit chow (5 g) to each tank. Tanks were seeded with 2 l of pond water from a nearby ephemeral pond 2 days before tadpoles were added. Once tadpoles were free swimming (Gosner stage 25), we added 40 tadpoles to each tank (10 randomly selected from each egg mass).

We weighed, measured and staged 10 tadpoles per tank at 2 months after the start of the experiment. In addition, we added 5 g of additional rabbit chow to each tank once per month. We placed floating foam pads in each tank to ensure metamorphosing froglets would not drown and we checked tanks daily to remove metamorphic animals with front limbs.

We assigned tanks according to a randomized block design to ensure equal spread across rows. We assigned each tank to one of two warming treatments (ambient or warmed) and one of three drying treatments [constant, slow (dried 2.5 cm in depth per week) and fast (dried 5 cm in depth per week)] in a fully factorial design. The ‘slow’ drying rate was chosen to result in a dry-down time of 112 days, which was sufficient to allow metamorphosis based on the published range of larval periods for this species (60–90 days; Ryan and Winne, 2001), whereas the ‘fast’ drying rate, resulting in a dry-down time of 63 days, could be representative of more extreme drying rates under climate change. We did not dry mesocosms lower than 3 cm in depth, and we held water depths at that level for the remainder of the experiment after reaching that point. Similar to other published mesocosm warming experiments (Davenport et al., 2017; O’Regan et al., 2014), we installed two aquarium heaters (Aqueon Pro, 100W, Franklin, WI, USA) in each warmed tank, set to 27.8°C, which resulted in a 2.3°C average increase in water temperature in comparison to ambient tanks. This aligns with predicted increases in air temperature for this region by the year 2070 (see Supplementary Materials and Methods, Future temperature modeling, Table S1). We lowered the installed standpipes to the desired water depth each week to achieve drying at the appropriate rate. We covered standpipe openings with mesh to ensure no tadpoles, rabbit chow or leaves were removed during drying. We checked water depth, animal health and water temperature daily, and monitored water quality weekly with a pH meter (Oakton PC Testr 35; pH, conductivity, total dissolved solids) and water test strips (Tetra EasyStrips for ammonia, nitrate, nitrite, hardness, chlorine and alkalinity). To monitor water temperature, two temperature loggers (HOBO UA-002-64, Onset Computer Corporation, Bourne, MA, USA) were installed 5 cm below the water surface, and 5 cm above the tank floor (see Supplementary Materials and Methods, Temperature variation in mesocosms, Fig. S1).

Animals removed from mesocosms were placed individually in 2.1 liter ventilated plastic containers with ~100 ml of mesocosm water, which we tilted to provide a dry refuge. These were transported to a field laboratory where they were kept at room temperature (20–23°C) for 1–3 weeks until tail resorption (Gosner stage 46). We then shipped juvenile frogs to the University of Pittsburgh where they were kept in the same enclosures at 19–21°C

and on a 14 h:10 h light:dark cycle. Given the range in larval periods, we split frogs into groups so that they were tested at similar ages ( $\pm\sim 2$  weeks) post-metamorphosis for performance and critical limit tests. We used one subset of frogs for jumping performance and critical temperature tests, and another for behavioral and thermal preference trials (tests performed in the order listed).

Permission to collect eggs was granted by the Louisiana Department of Wildlife and Fisheries Scientific and Collecting Permit LHNP-18-005, and this research was conducted according to the University of Pittsburgh Institutional Animal Care and Use Committee protocol IML-18052950.

### Thermal sensitivity of jumping performance

Starting 1–2 months (mean: 38 days, range: 26–54 days) post-metamorphosis, we assessed the jumping performance of metamorphic animals ( $N=101$ , 11–16 per treatment) at five test temperatures ranging from 8 to 32°C in a randomized order (20, 14, 8, 26 and 32°C), repeating the initial test temperature at the end to ensure animals maintained 90% of initial performance. Animals were placed within a walk-in environmental room (Darwin Chambers, St Louis, MO, USA) the night before testing. To begin a trial, we increased or decreased the temperature in the room by 1°C every 15 min until reaching the testing temperature, after which animals acclimated to the test temperature for 1 h. We tested each animal at every temperature, with two tests per day and at least 8 h of recovery time in between tests. We placed frogs in an 86×47.6×17.8 cm (length×width×height) arena and encouraged them to jump with gentle prodding of a gloved finger. Each frog jumped three times per temperature, with 5–10 min in between each jump for recovery.

### Critical temperatures

One week after jumping trials, we tested  $CT_{\min}$  ( $N=79$ ) and  $CT_{\max}$  ( $N=62$ ). To do so, we placed animals in 50 ml conical tubes with an air hole in the lid to ensure adequate ventilation. We added 5 ml of room temperature filtered tap water to the tubes and then placed them horizontally to float in an insulated water bath. We then raised/lowered temperature by 1°C per minute by adding heated or chilled water. This rate of temperature increase is common in the amphibian literature (Pottier et al., 2022b; Simon et al., 2015; Sunday et al., 2019; von May et al., 2019) and minimizes stress owing to the prolonged heat exposure that occurs during slower ramping rates (Sørensen et al., 2013). Each minute we tested the frog for righting reflex, in which the frog was flipped onto its back and allowed to right itself. When righting reflex ceased, we measured frog body surface temperature with a temperature probe (Temperature Tester 343, Test Products International, Beaverton, OR, USA) pressed to the ventral surface, after which we placed the frog in room temperature water to recover. Body surface temperature is highly correlated with core body temperature in small-bodied frogs (Rowley and Alford, 2007), thus we refer to this measurement as ‘body temperature’ throughout. We monitored bath temperature with a thermometer (Cook N Cool digital thermometer, Cooper Atkins, Middlefield, CT, USA), validated with a temperature data logger (HOBO UA-002-64, Onset Computer Corporation) inside a conical tube containing 5 ml of water and no frog. All animals recovered after these trials.

### Open-field behavioral test

We tested frogs ( $N=65$ ) in an open-field test, in which activity and behavior are monitored in an ‘open field’ or arena, to measure exploratory behavior in a novel environment (Carlson and

Langkilde, 2013; Kelleher et al., 2018). Animals underwent the test at 3–5 months post-metamorphosis (mean: 123 days, range: 109–157 days) at room temperature (20–21°C). The uncovered, circular arena (51 cm diameter; 50.8 cm high walls to prevent escape) was made of copper-coated aluminum sheeting painted matte white with aquarium-safe paint to reduce glare when filming. The floor of the arena was evenly covered with two low-lint towels (KimTech Science Kimwipes, 41×36 cm) saturated with 250 ml of filtered water. We placed frogs under a small dish in the center of the arena and then released them to explore the arena for 20 min, during which the trial was filmed from above using a Canon Powershot camera (SD850 IS, Canon USA). We conducted trials during daylight hours (09:00–18:00 h) and observers left the room during trials to minimize disturbance.

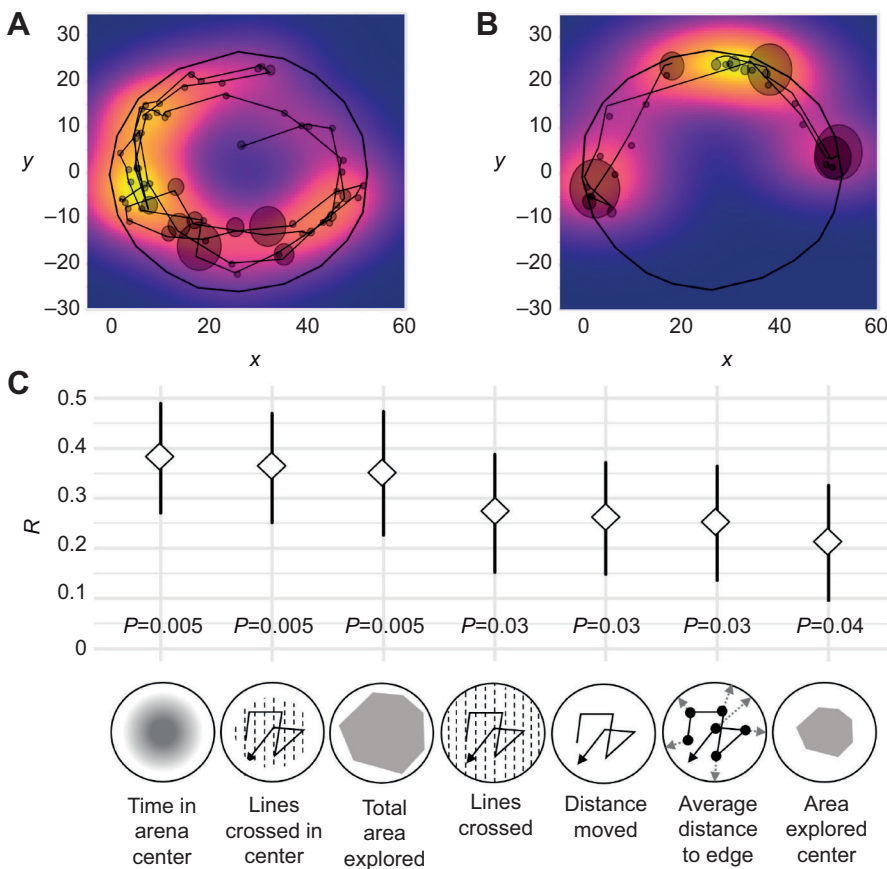
### Thermal preference test

At 6–7 months post-metamorphosis (mean: 204 days, range: 191–217 days), we tested a subset of the same animals from the behavioral trials ( $N=52$ ) in the same open field arena modified with a thermal gradient to assess thermal preference, following the methods of Anderson and Andrade (2017), with some modifications. A heating mat was placed under the arena at one edge (Zilla Heat Mat, 24 W) and plastic tubing containing water chilled by an aquarium chiller was placed under the opposite edge (AquaEuroUSA, model MC-1/4HP) to create a gradient of temperatures that ranged from  $\sim 15$  to 25°C. There was some variation in the thermal gradient temperatures across trials, but there was no significant difference in arena temperature across treatments [linear model (LM): maximum:  $F=1.26$ ,  $P=0.30$ ; minimum:  $F=0.25$ ,  $P=0.94$ ]. We filmed the 180 min trials from above with a video camera (YI 4 K Action Camera, Shanghai, China) and a thermal camera (FLIR model E40, 0.07°C thermal sensitivity, Wilsonville, OR, USA), to capture both frog behavior and body temperature. During trials, room temperature and humidity was recorded in order to correct images for any environmental distortion (Tattersall, 2016), and the camera remained at a fixed distance from the arenas. Using the last hour of the trial, to allow 2 h of acclimation/temperature selection, we determined frog body temperature every 60 s using FLIR ResearchIR thermal analysis software.

### Assessing behavioral traits

The beginning of the thermal preference trial was used in combination with the shorter behavioral trials to assess repeatability of behavioral traits (see ‘Statistical analyses’ below). We use the terms ‘behavioral traits’ or ‘behavioral phenotypes’ (rather than ‘personality’) in describing our findings because this study includes only one type of behavioral assay in one context (Carter et al., 2013).

We used the MTrackJ plugin (<http://www.imagescience.org/meijering/software/mtrackj>) in ImageJ to score behavioral trials, using modified methods from Hammond et al. (2020a). Briefly, the observer first selected a set of calibration points, including five identifiable, standardized locations in each video and 20 points indicating the arena’s edge. The observer then manually selected the location of the frog each time it moved during video playback. The program generated time-stamped  $x$ - and  $y$ -coordinates of the frog’s movements in each trial (Fig. 1A). For analyses, we cropped these data to 600 s for each individual because after this time point inter-individual differences in behavior were masked by the fact that most individuals stopped moving about the arena.



**Fig. 1. Example movement paths and repeatability estimates for behavioral traits.** Example movement path of (A) a frog with high activity that spent more time farther from the edge of the arena, and (B) another with lower activity and exploratory behavior. The black line represents the movement path for each frog. Circular point size indicates the length of time the frog spent at each location. The circular arena edge is shown as the outermost black line. The arena is shaded as a heat map, indicating how much the frog moved in a given area (yellow: more; blue: less). (C) Repeatability estimates ( $R$ ,  $\pm$ s.e.) for the seven behavioral traits quantified from each open-field test; false discovery rate adjusted  $P$ -values and a graphical depiction of the trait are shown below each datapoint. Figure builds on design and code from Hammond et al. (2020a).

For each trial, we calculated seven behavioral summary measures based on the coordinate data (Table 1, Fig. 1B). For measures of the ‘edge’ and ‘center’ of the arena, we defined the edge as any location within 6 cm of the arena wall [approximately twice the average snout–vent length (SVL) of the frogs]. For measures of area, we calculated the minimum convex polygon areas from movement tracks in R (package: adehabitatHR, function: mcp; Calenge, 2011). To quantify exploration, we divided arenas into 20 evenly distributed, vertical sections and calculated the number of section lines crossed during the trial (Hanson, 2013). We describe frogs as more ‘exploratory’ when they crossed more lines and explored larger areas (in the entire arena and/or arena center), spent more time in the center of the arena, and had greater average distances from the edge of the arena. In the personality literature, there is some variation and crossover in which measures are used to describe animals’ exploration tendencies, activity and boldness; other studies have used similar measures to the ones we use here to describe animals as exploratory, but also as more active or bold (Carlson and Langkilde, 2013; Kelleher et al., 2018; Rangassamy et al., 2015; Yuen et al., 2017).

### Statistical analyses

We conducted all statistical analyses in the R programming environment (<https://www.r-project.org/>). Models were fit with maximum likelihood and likelihood ratio tests (LRTs) were performed for model comparison of nested models (package: base, function: anova), whereas Akaike’s information criterion (AIC) was used when non-nested model comparison was required. Model residuals of all LMs followed a normal distribution, and we used the DHARMA package (<https://CRAN.R-project.org/package=DHARMA>) to verify model assumptions were met.

To test for differences in time to and size at metamorphosis, we performed a series of linear mixed-effects models (LMM) with treatment as a fixed effect, and mesocosm as a random effect (package: lme4, function: lmer). We used a generalized linear mixed model (GLMM) with a binomial distribution to assess differences in tadpole survival throughout the duration of the mesocosm period (package: lme4, function: glmer). The response variable was a two-column matrix composed of the number of surviving and dead individuals in each mesocosm. Larval period (days) was included as an interactive covariate in models

**Table 1. Ethogram showing the behavioral traits that were quantified from open-field test data**

Trait	Definition (units)	Transformed prior to analysis?
Time in center	Time in arena center (>~2 body lengths from edge) (s)	log
Lines crossed in center	Total number of gridlines crossed in arena center	Square root
Total area explored	Minimum convex polygon for entire movement track	Squared
Gridlines crossed	Total number of gridlines crossed	Square root
Total distance	Total distance moved (cm)	Square root
Distance to edge	Average distance to arena edge over entire trial (cm)	log
Area explored in center	Minimum convex polygon for entire movement track in center of arena only	Square root

**Table 2. Principal components analysis results for behavioral traits for the short trials**

Parameter	PC1	PC2	PC3	PC4	PC5	PC6
Lines crossed – center	–0.5028	0.263	–0.1179	0.4578	–0.3348	0.5852
Lines crossed	–0.1034	0.6663	0.0969	0.4146	0.2592	–0.5448
Area explored	0.0564	0.6529	–0.1589	–0.6868	–0.2427	0.1214
Time in center	–0.5087	–0.0805	–0.5015	–0.2253	0.6557	0.0495
Distance to edge	–0.5169	–0.2325	–0.1531	–0.1361	–0.5497	–0.5785
Area explored – center	–0.4554	–0.007	0.8225	–0.2786	0.1721	0.094
s.d.	1.7265	1.4097	0.72598	0.5027	0.40369	0.29867
Eigenvalue	2.9808	1.9872	0.5270	0.2527	0.1630	0.0892
Proportion of variance	0.4968	0.3312	0.08784	0.04212	0.02716	0.01487

PC1 was used for subsequent analyses involving behavior.

examining size at metamorphosis [separate models for SVL (mm) and mass (g)].

To test for differences in critical temperatures, we performed separate LMMs for  $CT_{min}$  and  $CT_{max}$ , and report the best supported model given LRT results, which include treatment, larval period and their interaction, as fixed effects in the  $CT_{min}$  model, and only larval period in the  $CT_{max}$  model.

We tested for the thermal dependence of jumping performance using a LMM with a second-order polynomial term to account for the expected non-linear relationship between temperature and performance (Huey and Stevenson, 1979). We used the maximum jump distance for each individual at each test temperature, and the model with the lowest AIC included individual ID nested in test temperature as a random effect to take into account repeated measures on the same individuals, and the residuals of femur length scaled to SVL as a covariate to account for variation in frog body morphometrics.

To assess repeatability, we used data from the short (10 min) behavioral trials along with the first 10 min of data from the longer, thermal preference trials, which were conducted in the same arena. We used the rptR package, which implements LMMs, to estimate the repeatability (intra-class correlation coefficient) of individual identity for each behavioral trait (Table 1; Stoffel et al., 2017). We included trial number (first or second) as a fixed effect and individual identity as a random effect. Traits were transformed prior to analysis (Table 1) in order to meet LMM assumptions (normality/homoscedasticity of residuals, etc.).

To control for multicollinearity between behavioral traits, which would violate the assumption of independent covariates in linear models, we applied principal components analysis (PCA) to the traits. We included all behavioral traits (Table 1) in the PCA except for distance moved, which was redundant with lines crossed (correlation test  $r=0.99$ ). We conducted separate PCAs for behavioral data from the short trial and for behavioral data from the last hour of the longer thermal preference trial and used the first PC (which accounted for ~50–57% of variance in the behavioral data; Tables 2, 3) in the relevant models (see below). Individuals with higher values of PC1 generally had lower exploration tendency, as we have defined it, i.e. individuals with higher values of PC1 crossed fewer lines (at the arena center and/or in the whole arena), spent less time and explored smaller areas in the arena center, and stayed closer to the arena edge. The area of the arena explored loaded less heavily on PC1 for the short behavior trials than the longer thermal preference trial (see Tables 2, 3).

We used LMMs to test for relationships between developmental treatments and other measured endpoints. Specifically, we used models to characterize the impacts of: (1) developmental treatment (warming/drying) on behavior; (2) developmental treatment on thermal preference; (3) behavior (prior to and during the thermal

preference tests) on thermal preference. We included SVL as a fixed effect and mesocosm identity as a random effect in all models. We always included an interaction term between drying and warming treatment in the starting model, but for simplicity eliminated it when it did not appear to explain meaningful variation in the data (i.e. when a LRT and AIC comparison suggested that it did not improve the model). In the model of behavior, we used as the response variable the first principal component from the behavioral data (Table 3), which aligned positively with animals that were less exploratory, and we included behavioral trial date as a random effect. We removed one outlier from analyses whose behavioral traits in the short trial (PC1) were over two standard deviations away from the mean and who appeared as an outlier in residuals plots, violating model assumptions. We used the visreg package (Breheny and Burchett, 2017) to generate partial residuals plots. We used LRTs to assess the significance of random effects.

## RESULTS

### Effects of drying and warming on survival, larval period and size at metamorphosis

Individually, fast drying (GLMM:  $\beta=-1.13$ ,  $P=0.017$ ) and, marginally, warming (GLMM:  $\beta=-0.90$ ,  $P=0.062$ ) decreased tadpole survival odds. However, the combination of warming and fast drying increased tadpole survival odds (GLMM:  $\beta=1.75$ ,  $P=0.0079$ ; Table S2). Both warming (LMM:  $\beta=-7.79$ ,  $P=0.0021$ ) and fast drying (LMM:  $\beta=-16.6$ ,  $P<0.001$ ) decreased larval period, with animals in warmed treatments metamorphosing on average 8.9 days earlier than ambient treatments, and those in fast-drying treatments metamorphosing on average 17.5 and 13.1 days earlier than the constant or slow treatments, respectively (Fig. 2A; Table S2). In addition, drying, but not warming, resulted in significantly smaller frogs at metamorphosis (LMM: mass, fast drying:  $\beta=-0.15$ ,  $P<0.001$ , slow drying:  $\beta=-0.17$ ,  $P<0.001$ , warming:  $\beta=0.02$ ,  $P=0.49$ ; SVL, fast drying:  $\beta=-2.32$ ,  $P<0.001$ , slow drying:  $\beta=-2.26$ ,  $P<0.001$ , warming:  $\beta=0.20$ ,  $P=0.54$ ; Fig. 2B; Table S3). Furthermore, larval period was non-linearly associated with size at metamorphosis, with early and late metamorphosing animals emerging at larger sizes than those with moderate larval periods (LMM: mass, larval period<sup>2</sup>:  $\beta=0.026$ ,  $P=0.004$ ; SVL, larval period<sup>2</sup>:  $\beta=1.03$ ,  $P<0.001$ ; Fig. 2B; Table S3).

### Carry-over effects of the developmental environment on critical temperatures

$CT_{min}$  increased with larval period (LMM:  $\beta=0.79$ , partial  $R^2=0.21$ ,  $CI=0.067-0.391$ ,  $P<0.001$ ) in all treatments except the fast-drying treatment (fast drying $\times$ larval period:  $\beta=-0.86$ , partial  $R^2=0.002$ ,  $CI=0.001-0.083$ ,  $P=0.015$ ; Fig. 3A; Table S4).  $CT_{max}$  was associated with larval period regardless of treatment (LMM:  $\beta=0.17$ , partial  $R^2=0.122$ ,  $CI=0.010-0.316$ ,  $P=0.008$ ; Fig. 3B;

**Table 3. PCA results for behavioral data from the thermal preference trials**

Parameter	PC1	PC2	PC3	PC4	PC5	PC6
Lines crossed – center	-0.5202	0.0401	-0.0128	0.3662	-0.486	0.5978
Lines crossed	-0.4721	0.3623	0.1311	0.1372	-0.2674	-0.7337
Area explored	-0.3998	0.4837	0.2446	-0.545	0.4069	0.2895
Time in center	-0.4063	-0.4735	0.257	0.4099	0.6095	-0.0719
Distance to edge	-0.3215	-0.638	0.0048	-0.6178	-0.3095	-0.1101
Area explored – center	-0.2798	0.0439	-0.9256	-0.0191	0.244	-0.0562
s.d.	1.8459	1.1710	0.9193	0.45397	0.32508	0.25387
Eigenvalue	3.4073	1.3712	0.8451	0.2061	0.1057	0.0644
Proportion of variance	0.5679	0.2286	0.1409	0.03435	0.01761	0.01074

PC1 was used in subsequent analyses.

Table S4), with frogs with a longer larval period able to withstand higher temperatures. The magnitude of the effect of larval period on  $CT_{min}$  was greater than on  $CT_{max}$ ; across a 2-month range in larval period, there was a  $\sim 4^{\circ}C$  increase in  $CT_{min}$ , but only a  $\sim 1^{\circ}C$  increase in  $CT_{max}$ . There was no association between warming treatment and critical temperatures (Table S4). In addition, we found no association between frog size and critical thermal limits, likely because of the low size range variation in these frogs of similar ages (Table S4).

### Thermal sensitivity of jumping performance and thermal performance curve

Twenty-one animals were removed from analyses because they did not jump at 90% or higher of their original jump distance on the repeated trial at  $20^{\circ}C$  (final  $N=80$ ). Maximum jump distance was positively associated with the residuals of  $SVL \sim femur$  length, thus these size residuals were included as a covariate in analyses to account for this relationship (LMM:  $\beta=2.160$ ,  $P<0.001$ ,  $CI=1.106-3.214$ ). Overall, animals from the ambient temperature, constant water level treatment jumped further at higher temperatures than all other treatments [LMM:  $treatment \times body\ temperature^2$ :  $F=11.158$ ,  $d.f.=6$ ,  $95.109$ ,  $P<0.001$ , partial eta squared ( $\eta_p^2$ )=0.41,

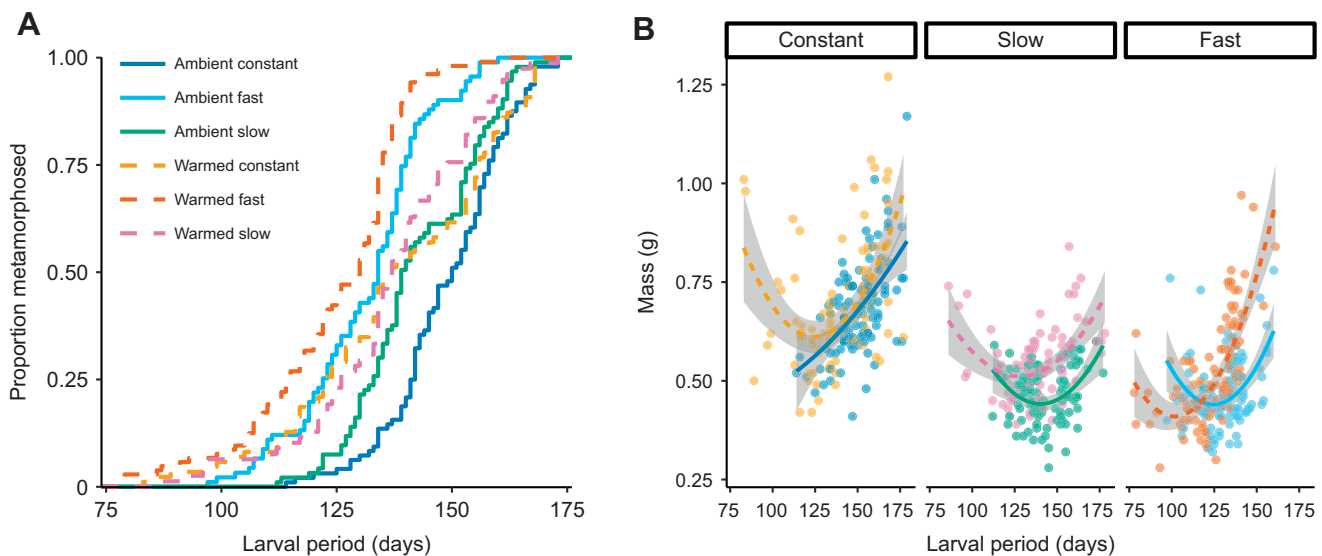
$CI=0.27-1.00$ ; *post hoc* interaction contrast (package: phia, function: testInteractions):  $\chi^2=6.73$ ,  $P=0.0095$ ; Fig. 4, Table S5; see Supplementary Materials and Methods, Thermal dependence of jumping performance, Table S6]. On average, animals in the ambient constant treatment jumped 12% further than all other treatments at the highest test temperature ( $32^{\circ}C$ ).

### Repeatability of behavioral traits

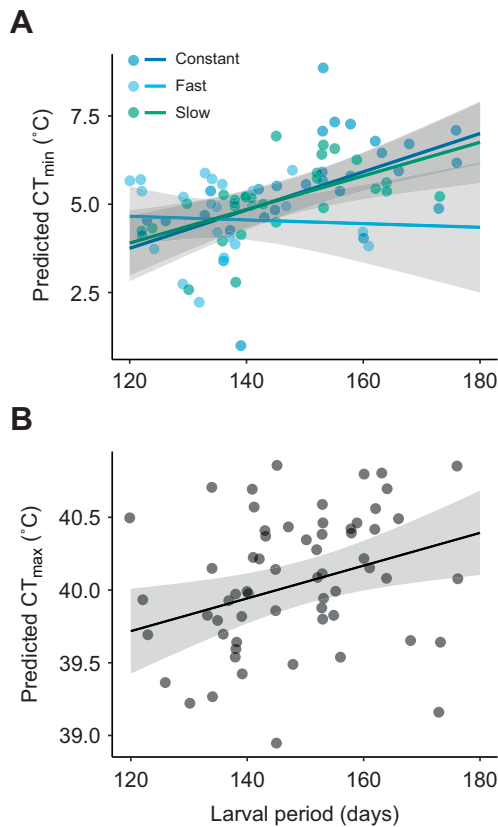
All of the behavioral traits we assessed were significantly repeatable at different levels (Fig. 1C). The time spent in the center, lines crossed in the centers and total area explored were the most repeatable traits ( $R>0.3$ ; Fig. 1C).

### Developmental environment and behavioral traits

Developmental treatment had a significant impact on behavioral traits expressed in the shorter behavioral trials (Table S7; Fig. 5). Specifically, there was an interactive effect between drying and warming treatments, such that frogs that experienced both fast drying and warming during development exhibited distinct behavioral traits in the short behavioral trial (LMM:  $\beta=2.93$ ,  $P=0.004$ ,  $CI=0.94-4.93$ ). These frogs had higher values of PC1, indicating that they were less exploratory (Table 2).



**Fig. 2. Effect of developmental treatment (drying and/or warming) on time to and size at metamorphosis in *Rana sphenocephala*.** (A) Time to metamorphosis; (B) size at metamorphosis (mass, g). Grey bands represent 95% confidence intervals, points represent individual animals. Mesocosms were stocked with 40 animals each ( $N=120$  per treatment), but the total that metamorphosed successfully from each treatment varied (ambient constant:  $N=101$ , ambient fast:  $N=91$ , ambient slow:  $N=94$ , warmed constant:  $N=87$ , warmed fast:  $N=104$ , warmed slow:  $N=81$ ). Both warming and fast drying increased development rate (LMM,  $P<0.001$ ), and only drying decreased size at metamorphosis (LMM:  $P<0.001$ ).



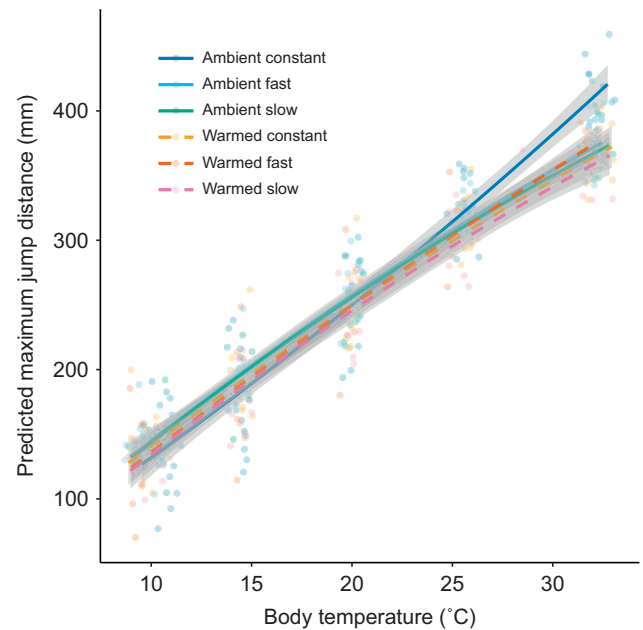
**Fig. 3. Model predictions of the effect of larval period and drying treatment on critical thermal limits in *Rana sphenocephala*.** (A) Critical thermal minima ( $CT_{min}$ ) increased with larval period (LMM,  $P < 0.001$ ,  $N = 79$ ), except in the fast-drying treatment (LMM,  $P < 0.01$ ,  $N = 79$ ), and (B) critical thermal maxima ( $CT_{max}$ ) increased with larval period (LMM,  $P < 0.01$ ,  $N = 62$ ). Grey bands represent 95% confidence intervals. Darker circles depict overlapping datapoints.

### Predictors of thermal preference

Developmental treatments were significantly predictive of thermal preference (Fig. 6; Table S8A). Specifically, both drying treatments (LM: slow drying:  $\beta = 0.98$ ,  $P = 0.006$ ,  $CI = 0.29\text{--}1.67$ ; fast drying:  $\beta = 0.73$ ,  $P = 0.040$ ,  $CI = 0.03\text{--}1.43$ ), but not the warming treatment (LM:  $\beta = -0.15$ ,  $P = 0.589$ ,  $CI = -0.72\text{--}0.42$ ), were predictive of thermal preference, with frogs from drying mesocosms exhibiting warmer temperature selections (Table S8A; Fig. 6A,B). Larger animals also selected warmer temperatures (LM:  $\beta = 0.65$ ,  $P < 0.001$ ,  $CI = 0.32\text{--}0.97$ ; Table S8A, Fig. S2). Behavioral traits were significantly predictive of thermal preference (LM:  $\beta = 0.23$ ,  $P = 0.015$ ,  $CI = 0.05\text{--}0.41$ ). For behavioral traits measured in the short behavioral trials, frogs with higher values of PC1, which indicated a less exploratory behavioral phenotype, were more likely to select warmer temperatures (Table 3, Fig. 6A). Additionally, behavior during the last hour of the thermal preference trial predicted thermal preference: less exploratory frogs (with higher values of PC1; Table 2; LM:  $\beta = 0.15$ ,  $P = 0.045$ ,  $CI = 0.00\text{--}0.30$ ) had warmer selected temperatures (Fig. 6C; Table S8B).

### DISCUSSION

The developmental environment has immediate and long-term impacts on physiology and behavioral phenotypes. For organisms that undergo metamorphosis, such as amphibians, there is mixed evidence that the early environment can have lasting impacts on

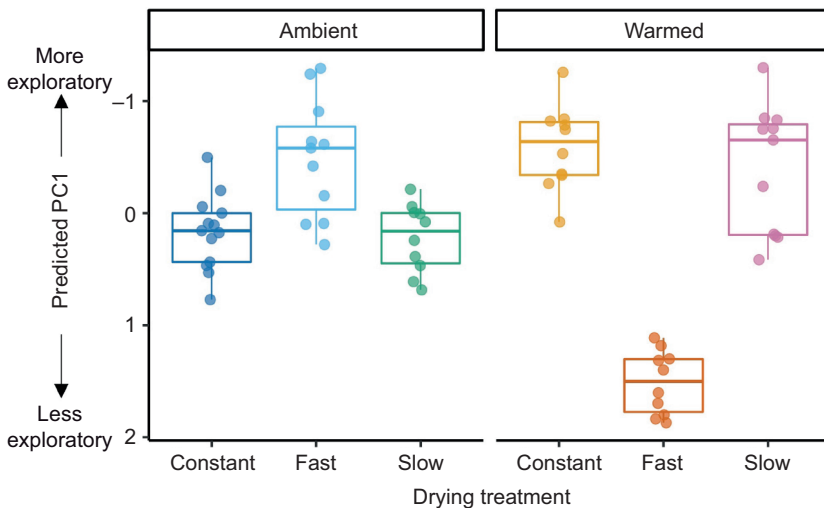


**Fig. 4. The modelled thermal dependence of jumping performance, controlling for the relationship between size and femur length (mm), across developmental treatments in *Rana sphenocephala*.** Animals from the ambient temperature, constant water level treatment jumped further at higher temperatures than all other treatments (LMM:  $P < 0.001$ ). Grey bands represent 95% confidence intervals. Sample sizes per treatment: ambient constant:  $N = 16$ , ambient fast:  $N = 13$ , ambient slow:  $N = 14$ , warmed constant:  $N = 11$ , warmed fast:  $N = 15$ , warmed slow:  $N = 11$ .

post-metamorphic phenotypes, and tests beyond the metamorphic stage are rare (reviewed in Stoks et al., 2022; Bodensteiner et al., 2021). We found that warming and drying conditions during the larval stage resulted in long-term impacts on thermal physiology and behavior in southern leopard frogs (*R. sphenocephala*). Larvae experiencing drying, and drying and warming together, accelerated development and metamorphosed at smaller sizes. The length of the larval period impacted critical temperatures into later life stages, and both drying and warming reduced jumping performance at high temperatures. Finally, the larval environment had lasting effects on both thermal preference and behavioral phenotypes, and behavioral phenotypes in a short open-field test were predictive of thermal preference in a thermal gradient. This work provides evidence that conditions during development can result in carry-over effects not only on morphology, but also on thermal physiology and behavior, which may have implications for amphibian survival in a changing world. Furthermore, this work highlights the importance of considering both behavior and thermal physiology concurrently, particularly in ectothermic organisms, as they are inextricably linked via behavioral thermoregulation.

### Early environment affects critical temperatures, thermal preference and performance

There is substantial evidence that developmental plasticity can impact critical thermal limits, often irreversibly (Kellermann et al., 2017; Schaefer and Ryan, 2006; Watkins and Vraspir, 2006). We believe this is the first work demonstrating a lasting impact of the length of the larval period, driven by pond drying, on critical temperatures. Animals with longer larval periods would have experienced higher absolute daily maximum and minimum water temperatures (because ambient temperatures rise throughout the



**Fig. 5. Box plots showing the predicted relationship between developmental drying/warming treatment and the first principal component (PC1) of behavioral data from the open-field behavioral trial (short trial, 10 min).** Frogs that experienced both fast drying and warming during development were less exploratory in the short behavioral trial (LMM;  $P < 0.001$ ). The boxes show the 25th–75th percentiles, whiskers are 1.5× the interquartile range, and the horizontal lines denote the median. Data points showing predicted values for PC1 are shown on top of the box-and-whiskers plots. Sample sizes per treatment: ambient constant:  $N=13$ , ambient fast:  $N=13$ , ambient slow:  $N=10$ , warmed constant:  $N=9$ , warmed fast:  $N=10$ , warmed slow:  $N=10$ .

spring and summer). Their developmental environment therefore paralleled their shift in higher  $CT_{min}$  and  $CT_{max}$ . Developmental plasticity in thermal physiology may be beneficial if it results in better matching to the resulting metamorphic environment (Beaman et al., 2016). Although previous work has found effects of developmental acclimation on adult critical thermal limits in *Drosophila* (Kellermann et al., 2017), future work should investigate whether these effects are reversible with adult acclimation in organisms with complex life cycles, and/or whether developmental plasticity increases adult acclimation capacity (van Heerwaarden et al., 2016; Beaman et al., 2016). Furthermore, animals from the slow- and fast-drying treatments preferred warmer temperatures in the thermal preference trials. Although a recent meta-analysis across ectotherms determined that early thermal environments had a weak overall impact on thermal tolerance later in life, very few studies examined lasting impacts across life stages, and developmental plasticity was three times higher in aquatic ectotherms, such as amphibians (Pottier et al., 2022a). This work provides evidence that a warming, drying pond would likely result in animals with shorter larval periods and shifted thermal performance windows, which could have direct consequences for withstanding a warmer, more variable world.

In addition, we found that elevated temperatures and pond drying during development reduced jumping performance at high temperatures later in life. Anurans rely on jumping not only to escape predators and capture prey, but also to disperse to new habitats. Jumping distance is heavily temperature-dependent (Mitchell and Bergmann, 2016; Putnam and Bennett, 1981; Wassersug and Sperry, 1977). Previous work has found lasting carry-over effects of developmental temperature (Álvarez and Nicieza, 2002; Drakulić et al., 2016; Orizaola and Laurila, 2009) and drying (Charbonnier and Vonesh, 2015; Richter-Boix et al., 2006) on amphibian jumping performance, often mediated via impacts on size at metamorphosis and hindlimb length. In addition, there is evidence that developmental temperature and daily temperature variation can modify the thermal dependence of performance (Měráková and Gvoždík, 2009; Orizaola and Laurila, 2009; Scott and Johnston, 2012). Our results align with previous work, indicating that drying and warming can be detrimental to post-metamorphic performance. We found only a slight increase in performance in the ambient temperature, constant water level treatment at the highest test temperature. Although compensatory growth could ameliorate some impacts of reduced

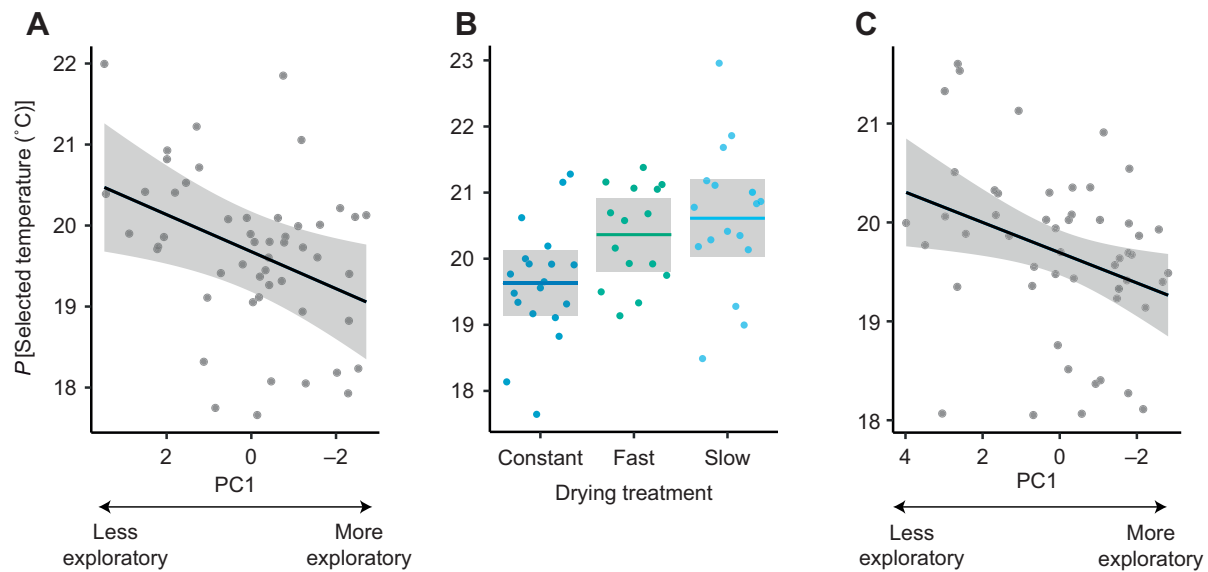
size at metamorphosis on size-dependent performance later in life (such as jumping distance), this compensatory growth could result in trade-offs with metabolic efficiency, particularly at high temperatures (Burraco et al., 2020). If increasing temperatures and environmental change during development reduce performance at high temperatures later in life, the negative effects of climate change for amphibians could be compounded across life stages.

#### Relationships between behavior and thermal biology

In comparison to other vertebrate species, amphibians have been understudied in the animal behavior literature, particularly in terms of consistent behavioral phenotypes (Kelleher et al., 2018). This work adds to a growing literature suggesting that amphibians exhibit behavioral traits that differ between individuals while exhibiting consistency within individuals. Repeatability levels for the effect of individual identity on behavioral traits were low but comparable to other studies of amphibians and other vertebrate species (Bell et al., 2009; Brodin et al., 2013; Carlson and Langkilde, 2013; Gifford et al., 2014; Kelleher et al., 2017; Smith and Doupnik, 2005).

Behavioral traits were affected by hydro-thermal developmental treatment, with animals from the most extreme group that was exposed to both fast drying and warming exhibiting less exploratory phenotypes in comparison to other animals. This result is noteworthy in the context of climate change, which is expected to result in both warmer and drier climates in many parts of the world (Cook et al., 2018; Rahmstorf and Coumou, 2011; Vasseur et al., 2014). If environmental warming or drying has interactive impacts not just on physiological and morphological traits (Brannelly et al., 2019; Kohli et al., 2019; LaBarbera et al., 2020; Márquez-García et al., 2009), but also on behavioral traits that direct the manner in which animals interact with their environments and with each other (Hammond et al., 2020b), it is possible that the impacts of climate change may be amplified beyond what has been predicted. If the larval environment can impact behavioral phenotype and thermal preference, this may have far-reaching consequences for amphibians facing not only climate change, but also the effects of widespread pathogens (most notably *Batrachochytrium* fungi and ranaviruses). Less exploratory animals may be less likely to become infected (Koprivnikar et al., 2012), but also less likely to gain defensive bacterial symbionts (Keiser et al., 2019), and a selection for cooler temperatures may also increase susceptibility to disease (Sauer et al., 2018). Our results highlight the knock-on effects of the developmental environment and the challenge of





**Fig. 6. Relationship between thermal preference, behavior and the developmental environment.** Partial residuals plots show the relationship between thermal preference and (A) behavior during the short open-field test, (B) drying treatment and (C) behavior during the thermal preference trial. Frogs with higher values of PC1 in the short open-field test, which indicated a less exploratory behavioral phenotype, were more likely to select warmer temperatures (A; LM:  $P < 0.05$ ). Frogs from drying mesocosms exhibited warmer temperature selections overall (B; LM:  $P < 0.05$ ). Finally, behavior during the last hour of the thermal preference trial predicted thermal preference: less exploratory frogs (with higher values of PC1) had selected warmer temperatures (LM:  $P < 0.05$ ). Grey bands represent 95% confidence intervals. Sample sizes per treatment when comparing short open-field and thermal preference trials: constant:  $N = 19$ , fast drying:  $N = 15$ , slow drying:  $N = 17$ .

predicting how a changing climate will impact organisms with complex life cycles.

Although previous work has supported the idea that more active, exploratory or bold animals tend to exhibit higher thermal preferences (e.g. Cerqueira et al., 2016; Goulet et al., 2017a; Michelangeli et al., 2018; Rey et al., 2015), our results suggested the opposite: frogs that were less exploratory selected warmer temperatures. Previous studies have focused largely on lizards (Goulet et al., 2017b; Michelangeli et al., 2018) and fish (Cerqueira et al., 2016; Killen, 2014; Rey et al., 2015); thus, it is possible that the opposite relationship holds true in amphibians. However, at least one study in squamates found a similar relationship in which more exploratory/bold individuals selected cooler temperatures (Goulet et al., 2018).

In our open-field test, to attain a higher body temperature than room temperature in a thermal gradient with a wet substrate, a frog would have needed to select a warm location within the gradient for enough time to increase its body temperature. More exploratory animals, therefore, may not have selected a location in this way, resulting in a cooler body temperature. Thus, body temperature (used to estimate thermal preference) could have been a by-product of behavioral phenotype, rather than active selection, per se. This suggests that repeatable behavioral traits, such as exploratory tendencies, may be important to consider in the methodology of studies that rely on behavioral indicators to measure non-behavioral traits. Thermal preference trials such as the ones performed in this study often use an open-field test format, allowing animals to select a location/temperature within an open arena containing a temperature gradient (Mitchell and Bergmann, 2016; Taylor et al., 2021). If more ‘shy’ or less exploratory animals are hesitant to move about the arena, this could mean that they do not discover the thermal gradient or are unwilling to inhabit particular parts of it (Cerqueira et al., 2016; Killen et al., 2021; Rey et al., 2015). Future work disentangling the relationship between behavioral phenotype and temperature selection in free-ranging amphibians is necessary.

## Conclusions

This work provides striking evidence that the developmental environment can have lasting effects on not only morphology, but also thermal physiology, performance and behavior in animals with complex life cycles. Our results suggest that, for amphibians, the predicted effects of climate change, including higher temperatures and faster pond drying rates, may have long-term effects on populations and their ability to withstand future change. Furthermore, behavioral phenotype and thermal preference are linked in ectotherms, and a better understanding of how one may influence the other will lead to better predictions of both plastic and evolutionary responses to climate change.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: M.E.B.O., T.T.H., C.L.R.-Z.; Methodology: M.E.B.O., T.T.H., C.L.R.-Z.; Software: M.E.B.O., T.T.H.; Validation: M.E.B.O., T.T.H.; Formal analysis: M.E.B.O., T.T.H.; Investigation: M.E.B.O., S.S., T.W., J.G.B., E.P., J.C.; Resources: C.L.R.-Z.; Data curation: M.E.B.O., T.T.H., S.S., T.W., J.G.B., E.P., J.C.; Writing - original draft: M.E.B.O.; Writing - review & editing: M.E.B.O., T.T.H., S.S., T.W., J.G.B., E.P., J.C., C.L.R.-Z.; Visualization: M.E.B.O., T.T.H.; Supervision: M.E.B.O., C.L.R.-Z.; Project administration: M.E.B.O.; Funding acquisition: C.L.R.-Z.

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## Data availability

All data supporting this article are available from the Dryad Digital Repository (Ohmer et al., 2023): <https://doi.org/10.5061/dryad.931zcrjqj>.

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