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RESEARCH ARTICLE

Prolonged survival out of water is linked to a slow pace of life in a self-fertilizing amphibious fish

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ABSTRACT

Metabolic rate and life-history traits vary widely both among and within species, reflecting trade-offs in energy allocation, but the proximate and ultimate causes of variation are not well understood. We tested the hypothesis that these trade-offs are mediated by environmental heterogeneity, using isogenic strains of the amphibious fish *Kryptolebias marmoratus* that vary in the amount of time each can survive out of water. Consistent with pace of life theory, the strain that survived air exposure the longest generally exhibited a 'slow' phenotype, including the lowest metabolic rate, largest scope for metabolic depression, slowest consumption of energy stores and least investment in reproduction under standard conditions. Growth rates were fastest in the otherwise slow strain, however. We then tested for fitness trade-offs between 'fast' and 'slow' strains using microcosms where fish were held either with constant water availability or under fluctuating conditions where water was absent for half of the experiment. Under both conditions the slow strain grew larger and was in better condition, and under fluctuating conditions the slow strain produced more embryos. However, the fast strain had larger adult population sizes under both conditions, indicating that fecundity is not the sole determinant of population size in this species. We conclude that genetically based differences in the pace of life of amphibious fish determine survival duration out of water. Relatively slow fish tended to perform better under conditions of limited water availability, but there was no detectable cost under control conditions. Thus, pace of life differences may reflect a conditionally neutral instead of antagonistic trade-off.

KEY WORDS: Metabolism, Life history, Trade-off, *Kryptolebias marmoratus*, Mangrove rivulus

INTRODUCTION

Life history and metabolic rate are often conceptually linked to form pace of life syndromes, in which organisms span a continuum of 'fast' (rapid growth, early age at maturity, high metabolism) to 'slow' (slow growth, delayed maturity, low metabolism) lifestyles (Ricklefs and Wikelski, 2002; Réale et al., 2010; Arnqvist et al., 2017; Auer et al., 2018). Pace of life varies substantially both among and within species, but the mechanistic causes and ultimate consequences of this variation are not well understood (e.g. Stearns, 1992; Burton et al., 2011; Careau and Garland, 2012). Variation between fast and slow lifestyles is thought to reflect trade-offs in energy allocation, for example to growth versus reproduction. Energy allocation depends first on energy acquisition and resource availability; thus, spatial or temporal environmental heterogeneity in resource availability is thought to be a key factor that produces and maintains variation in pace of life (Mueller and Diamond, 2001; Reid et al., 2011). However, the direction and mechanistic relationships between environmental conditions, life history and metabolic rate remain unclear (Koons et al., 2008; Burton et al., 2011; Auer et al., 2018).

Organisms that inhabit extreme and variable environments are useful systems for understanding the mechanisms that generate and maintain variation in pace of life (Passow et al., 2017). One of the most abrupt and dramatic changes in the physical environment experienced by any animal occurs when amphibious fishes move between water and land (Dejours, 1976; Graham, 1997; Wright and Turko, 2016). Among the many challenges faced by fish out of water, capturing and consuming prey is particularly problematic, as the low density of air makes suction feeding difficult (Heiss et al., 2018). Thus, most amphibious fishes must rely on internal energy stores when out of water, although there are exceptions (Heiss et al., 2018). The ability to deeply depress metabolism is thought to be a key factor enabling prolonged survival out of water in amphibious fishes such as the African lungfish *Protopterus aethiopicus*, which reduce O_2 consumption by about 80% while aestivating on land (Guppy and Withers, 1999). Many species do not aestivate, however, and the mechanisms that underlie variation in emersion tolerance are not well understood. In non-aestivating species, a relatively low overall metabolic rate would conserve resources (e.g. Killen et al., 2011) and could extend survival out of water. Thus, environmental variation in water availability may be a key factor that causes and maintains intra-specific variation in metabolism and life-history traits of amphibious fishes, but this hypothesis has not been tested.

Our objective was to understand the proximate and ultimate reasons why some amphibious fishes survive out of water longer than others. First, we tested the hypothesis that the amount of time amphibious fishes can spend out of water is limited by their pace of life. This hypothesis predicts that emersion-tolerant fishes will have relatively slow metabolic and growth rates, reduced consumption of energy stores, and low levels of activity and reproductive output. We then tested whether the costs and benefits of different paces of life ultimately depend on environmental conditions, i.e. whether there is an antagonistic trade-off between emersion tolerance and aquatic performance. Specifically, we investigated the prediction that a relatively 'fast' lifestyle would be favoured when food and water were constantly available, while a 'slow' pace of life would be favoured in a fluctuating environment frequently lacking water.

We tested these hypotheses using the amphibious mangrove rivulus *Kryptolebias marmoratus*, one of only two known

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Received 20 June 2019; Accepted 25 November 2019

self-fertilizing hermaphroditic vertebrates (Harrington, 1961; Avise and Tatarenkov, 2015) – the other is the sister species *Kryptolebias hermaphroditus* (Costa, 2011). This 'selfing' reproductive system allows the production of large numbers of isogenic, effectively clonal individuals and ultimately enables repeated experiments on the same genotype (Tatarenkov et al., 2010; Turko et al., 2011; Earley et al., 2012). *Kryptolebias marmoratus* can survive more than 66 days out of water in leaf litter or packed nose-to-tail within rotting mangrove logs (Taylor et al., 2008). There is no evidence that *K. marmoratus* aestivates when on land (Ong et al., 2007; Blanchard et al., 2019), but they are largely inactive (Turko et al., 2014, 2017) and do not eat (Pronko et al., 2013; Wells et al., 2015). We first measured energy use and oxygen uptake in isogenic strains acclimated to terrestrial conditions for 21 days. Then, fish from 'fast' and 'slow' strains (relatively high or low metabolic rate, estimated by rates of oxygen uptake) were reared together for 12 months in microcosms in which water was either always present or absent for random periods totalling 6 months, and the number, condition and reproductive output of the fish was measured.

MATERIALS AND METHODS

Animals

For all physiological experiments, *Kryptolebias marmoratus* (Poey 1880) hermaphrodites were raised individually in the Hagen Aqualab, University of Guelph, in 120 ml plastic holding cups. Fish from the isogenic strains 50.91 ('Belize', from Twin Cayes, Papa Gabriel, Belize), SLC8E ('Florida', from St Lucie County, FL, USA) and HON11 ('Honduras', from Bay Islands, Utila, Honduras) were used in these experiments (Tatarenkov et al., 2010). Fish were kept at 25°C, 15‰ salinity, with a 12 h:12 h light:dark cycle and were fed *Artemia* nauplii 3 times per week. Fish were fed to satiation for three consecutive days immediately prior to all experiments but were not fed for the duration of the experiments. Fish in microcosms were fed live *Artemia* nauplii 3 times per week but food was not added to fluctuating microcosms when water was not present. All experiments were approved by the University of Guelph animal care committee. Emersion tolerance, metabolic rate and energy use experiments were conducted in 2015–2016, microcosm experiments were conducted in 2016–2017, and genetic identification of these fish occurred in 2017–2018.

Emersion tolerance

To measure emersion tolerance, size-matched fish (n=20 per strain) were terrestrially acclimated on moist filter paper (Ong et al., 2007), and survival was monitored at least once per day (Wells et al., 2015). For ethical reasons, the experiment was terminated when 20% of each strain remained, as statistically significant differences among strains were clear at this point; remaining fish were killed with tricaine methanesulfonate (MS222; 500 mg l^{-1}).

Metabolic rate

To test the hypothesis that emersion tolerance depends on metabolic rate, O_2 consumption (n=8–10 per strain) was compared in water (control) and during air exposure using intermittent flow respirometry (Loligo Systems WITROX 4, Viborg, Denmark; 2.5 ml chambers carefully cleaned with ethanol prior to each trial) as described elsewhere (Livingston et al., 2018; Sutton et al., 2018) with the following modifications. Aquatic O_2 consumption of each individual was measured in triplicate (3 h chamber acclimation with flow-through normoxic water, 12–15 min recordings, 10 min flushing periods; measurements occurred between 11:00 h and 13:00 h), then water was drained from the chambers and aerial O_2 consumption was measured once per day for 7 days at the same time (12:00 h–16:00 h) to minimize the effect of diurnal metabolic rhythms (Rodela and Wright, 2006). Humidified air (100% relative humidity) was introduced into the chambers between measurements. Fish were weighed before and after the 7 day experiment and the average mass was used for statistical analyses and to standardize O_2 uptake (mean± s.e.m. mass of Belize fish 0.071±0.004 g, Florida fish 0.072±0.003 g, Honduras fish 0.076±0.003 g; no difference among groups P=0.64). To measure O_2 uptake after 3 weeks in air, an initial O_2 consumption rate in water was first determined for a separate group of fish, then fish were air exposed for 21 days and O_2 uptake was measured in air (n=10 per strain). For these fish, mass was measured immediately after each measurement of O_2 uptake (mass of Belize fish before 0.070±0.005 g, after 0.56±0.003 g; Florida fish before 0.078± 0.006 g, after 0.060±0.006 g; Honduras fish before 0.099±0.005 g, after 0.089±0.006 g; among strains P=0.001, before versus after P<0.001). Activity of the fish was not observed during these measurements. Microbial respiration, measured after each trial, was negligible.

Energy reserves and consumption

To test whether energy reserves and/or energy use were related to emersion tolerance, we measured activity, overall body condition and body composition. To measure activity, fish (n=8 per strain) were photographed every 5 s for 1 h (between 12:00 h and 13:00 h) at six time points (1 h, and 1, 3, 7, 14, 21 days out of water). Activity on land consists of discrete jumps, so activity was quantified as the proportion of photos in which fish had changed location between consecutive frames (Turko et al., 2014). Fulton's K, a general index of body condition (Froese, 2006), was calculated for control fish in water (n=6 per strain) and in a separate group of fish (n=12 per strain) that were terrestrially acclimated for 21 days. To measure body composition, independent groups of fish were required because the small size of *K. marmoratus* precluded measuring multiple energy reserves in the same sample. Fish held in water (control) or air (21 days) were killed (MS222), blotted dry, weighed and snap frozen in liquid nitrogen (for glycogen and protein determination) or dried (48 h at 50°C) for lipid analysis and measurement of water content. Glycogen content of whole fish (n=8–9 per strain) was measured enzymatically (Bergmeyer et al., 1974). Whole-body lipid stores (n=6–12 per strain) were measured by chloroform extraction (Junior and Peixoto, 2013). Crude protein (n=6–10 per strain) was measured using the Kjeldahl method (AOAC, 1995). Tecator Kjeltec digestion and distillation units (Foss, Eden Prairie, MN, USA) were used for protein analysis and the percentage of total nitrogen was determined based on a dry matter basis (%N×6.25) (Bureau et al., 2000). Body water content (n=6–12 per strain) was calculated by subtracting wet from dry body mass and dividing by the wet mass.

To calculate energy use during emersion, lipid, glycogen and protein utilization were each calculated by subtracting the average mass-specific energy stores (mg g^{-1}) in terrestrially acclimated fish (E_{terr}) from those of the control fish (E_{con}), accounting for changes in body water content (W) and the average change in body mass (ΔM_b), according to the formula:

$$\text{Energy consumed} = E_{con}(1 - W_{con}) - E_{terr}(1 - W_{terr})\Delta M_b. \quad (1)$$

All data used for these calculations are provided in Table S1. Total energy use (i.e. E_{con} and E_{terr} in Eqn 1) was calculated by adding the energy contained in the consumed glycogen (17 kJ g^{-1}), lipid (37 kJ g^{-1}) and protein (17 kJ g^{-1}). Overall standard deviations

were calculated using standard methods of error propagation, and effective degrees of freedom were calculated using the Welch–Satterthwaite equation (JCGM, 2008). Using these values, we compared energy use among the isogenic lineages with one-way ANOVA and *post hoc* Holm–Šidák tests. Data were ln transformed when necessary to meet assumptions of normality and equal variance.

Life history traits

Routinely collected records from our *K. marmoratus* colony were used to compare overall embryo production, clutch size and age at first reproduction among strains. To assess cumulative reproductive output, we only used data from fish that hatched within 1 year of those used for experiments, released at least one embryo, were never used for any experiments, and survived for over 18 months (Belize n=42, Florida n=27, Honduras n=20). Age at first reproduction was determined for a larger subset of fish that simply released an embryo prior to use in any experiments (Belize n=90, Florida n=72, Honduras n=90).

Microcosms

We used a 12 month microcosm experiment to test the hypothesis that there is an environmentally mediated trade-off between emersion tolerance and metabolic rate. Belize and Honduras fish (n=3 per strain per microcosm) were placed into each microcosm at the start of the experiment. Fish were size matched to minimize performance differences in aggressive/competitive interactions between the strains (Earley and Hsu, 2008), which resulted in Belize fish being slightly older (434±9.4 versus 325±17 days old) but of similar mass (Fig. S1) at the beginning of the experiment. All fish were sexually mature and had released at least one embryo in the laboratory colony before being placed in a microcosm to standardize reproductive status. We maintained constant water levels in control microcosms (n=10), while fluctuating microcosms (n=10) were drained and refilled (every 1–3 weeks, randomly assigned) such that water was absent for half of the experiment. Microcosms were constructed from 9 l plastic boxes (38×24×14 cm) filled half-way with 15‰ brackish water. The bottom was covered with soft filtration media to provide a moist substrate in the fluctuating treatment when water was absent, and 20 pieces of grey plastic pipe (3 cm length, 1.5 cm diameter) and three green acrylic yarn mops were added to provide shelter. Emersion periods never exceeded 3 weeks because our emersion tolerance data showed that 100% of fish survived this duration, and the goal of this experiment was to test whether sub-lethal effects could mediate a trade-off between fast and slow fish. Water levels were altered (fluctuating) or water was refreshed (control) via permanently installed plastic tubing under the filter media to minimize disturbance.

After the 12 month experiment, all adult fish were killed (MS222), photographed (for length measurements), weighed, and a piece of caudal fin tissue was fixed in DNA preservative (0.25 mol l^{-1} EDTA, 20% dimethyl sulfoxide, NaCl saturated, pH 7.5; Seutin et al., 1991) for determination of genetic identity. Gonads and liver were dissected and weighed to assess condition. Gills were removed to test whether gill surface area was related to pace of life differences. The number and length of gill filaments was measured in whole mounts of the left-side arches. Sex was assessed based on external morphology (Scarsella et al., 2018) and appearance of ovarian tissue in the gonads. All embryos were collected and fixed in DNA preservative (see above) for genetic identification. No larvae or juvenile fish were found.

The genetic strain (i.e. Belize or Honduras) of each adult and embryo was determined using previously described protocols and microsatellite markers (Mackiewicz et al., 2006; Tatarenkov et al., 2010). Genomic DNA was extracted and purified using a commercially available kit according to manufacturer's instructions (GeneJET DNA Purification Kit, Fisher Scientific). Microsatellite 'R18' from Mackiewicz et al. (2006) was used to differentiate between strains, as this is one of the three most divergent loci between Belize and Honduran fish and amplified more consistently than the other most divergent loci (R3 and R34). PCR products were run on an acrylamide gel (5%, 3000 V for 2 h at 55°C), and were manually scored for strain identity. We were able to identify 100% of the adult fish; however, some embryos (20 of 81 from control, 209 of 979 from fluctuating microcosms) could not be confidently assigned to either strain, probably due to low DNA content. These unidentified embryos were excluded from statistical analysis.

Statistical analysis

Survival out of water was compared among strains using a Kaplan–Meier survival analysis. Rates of oxygen consumption were compared among strains and over time using both ANOVA (comparison of mass-corrected rates) and a linear model that included body mass as a covariate (R package lme; http://CRAN.R-project.org/package=nlme). Energy stores and energy consumption was compared among strains using one- or two-way ANOVA as appropriate, followed by Holm–Šidák *post hoc* tests. Life-history data (embryo production, age at first reproduction) were not normally distributed and were therefore analysed using a Kruskal–Wallis ANOVA on ranks. Growth rates of Honduras versus Belize fish were compared using ANCOVA (dependent variables: length, mass; covariate: age). Body size, condition, and organ sizes of microcosm fish were compared using two-way ANOVA. Embryo production between strains and treatments was compared using ANCOVA to account for different numbers of adults in each microcosm at the end of the experiment. Critical α=0.05 for all tests; throughout the text, values are given as means±s.e.m.

RESULTS

Survival out of water was significantly longer in the Honduras strain relative to the Belize and Florida strains (Kaplan–Meier log-rank statistic=11.198, P=0.004; Fig. 1). The Honduras strain had consistently lower (by 30–50%) rates of O_2 consumption in both water and air relative to Belize and Florida strains over 7 days, both when mass-corrected rates were compared directly (ANOVA, $F_{2,24}$=9.96, P<0.001) and when body mass was included as a covariate in the statistical model (lme, χ^2=23.02, P<0.001; Fig. 2A). Overall, O_2 consumption increased in all strains after 2 and 3 days in air compared with the other time points (lme, χ^2=23.21, P=0.003, interaction P=0.20; Fig. 2A). In the 21 day terrestrial acclimation experiment, mass-specific O_2 consumption of Honduras fish was again lower (by ∼40%) than that of Belize and Florida fish. This difference was significant when mass-corrected values were compared with ANOVA ($F_{2,27}$=4.81, P=0.016), and approached statistical significance when mass was included as a covariate in the statistical model (lme, χ^2=4.34, P=0.11; Fig. 2B). This discrepancy between the different analyses is probably because the Honduras fish used in this experiment were slightly larger (∼30%) than the other strains (see Materials and Methods). After 21 days in air, rates of O_2 consumption decreased by 44% (lme, χ^2=19.85, P<0.0001, interaction P=0.45; Fig. 2B).

There was no difference in initial body condition among the three strains, but terrestrial acclimation for 21 days resulted in significantly lower condition factor of Belize and Florida fish, but not Honduras fish (two-way ANOVA $F_{2,47}$=3.35, interaction

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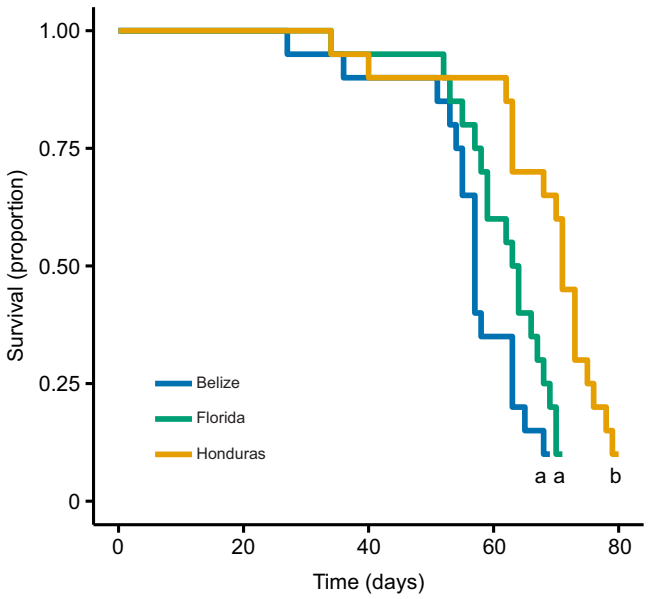

Fig. 1. Survival of three different strains of *Kryptolebias marmoratus* out of water. Different letters represent significant differences between strains ((Belize, Florida, Honduras; $P<0.05$).

$P=0.024$; Fig. 3). Similarly, in paired measurements of different individuals, Honduras fish lost significantly less body mass than other strains after 21 days out of water (ANOVA $F_{2,27}=6.92$, $P=0.004$; Fig. S2). All three strains in water had similar levels of glycogen, lipid and protein stores at the beginning of the experiment (two-way ANOVA, all $P>0.05$; Fig. S3). After 21 days out of water, there was no difference among strains in lipid use (ANOVA, $F_{2,83}=0.92$, $P=0.40$; Fig. 4A), but Florida fish used more glycogen than the other strains (ANOVA, $F_{2,191}=6.72$, $P=0.002$; Fig. 4B). Florida fish also consumed the most protein; Honduras fish consumed the least (ANOVA, $F_{2,779}=47.60$, $P<0.001$; Fig. 4C). Overall, Honduras fish used significantly less energy over 21 days on land compared with Belize and Florida fish (ANOVA, $F_{2,65}=5.21$, $P=0.008$; Fig. 4D). Water content was significantly elevated in all strains after 21 days on land (two-way ANOVA, $F_{1,53}=75.31$, $P<0.001$, interaction $P=0.60$; Fig. S2D). Activity generally decreased over 21 days out of water, but differed among the strains only at the 1 h time point (two-way ANOVA $F_{2,16}=5.67$, interaction $P=0.001$; Fig. S4).

In long-term studies of our laboratory colony, Belize fish produced more embryos (Kruskal–Wallis ANOVA on ranks, $H_2=10.61$, $P=0.005$; Fig. 5A) and had larger mean clutches (Kruskal–Wallis ANOVA on ranks $H_2=20.12$, $P<0.001$; Fig. 5B) than Florida or Honduras fish over the first 18 months of life, while age at first reproduction tended to be earlier (Kruskal–Wallis ANOVA on ranks, $H_2=6.91$, $P=0.032$; Fig. 5C).

Belize and Florida fish generally showed similar trends, so we focused on a comparison of Belize and Honduras fish for our follow-up microcosm experiments. At the beginning of these experiments, Honduras fish were heavier (ANCOVA, $F_{1,287}=163.75$, $P<0.0001$; Fig. S1A) and longer (ANCOVA, $F_{1,287}=83.60$, $P<0.0001$; Fig. S1B) than Belize fish at a given age, indicating a faster growth rate.

At the end of the microcosm experiment, there was no significant difference in the total number of fish between control and fluctuating microcosms ($F_{1,39}=0.10$, $P=0.76$), but there were significantly more Belize than Honduras fish overall ($F_{1,39}=7.92$, $P=0.008$, interaction $P=0.76$; Fig. 6A). Honduras fish were longer (two-way ANOVA, $F_{1,110}=49.95$, $P<0.001$; Fig. 6B), heavier (two-way ANOVA, $F_{1,110}=80.40$, $P<0.001$; Fig. 6C), and in better condition (Fulton's K, two-way ANOVA, $F_{1,110}=36.24$, $P<0.001$; Fig. 6D; Fig. S5A). Gonado-somatic index was also significantly higher in Honduras fish (two-way ANOVA, $F_{1,110}=93.76$, $P<0.001$; Fig. 6E; Fig. S5B), but there was no difference in hepato-somatic index between the two

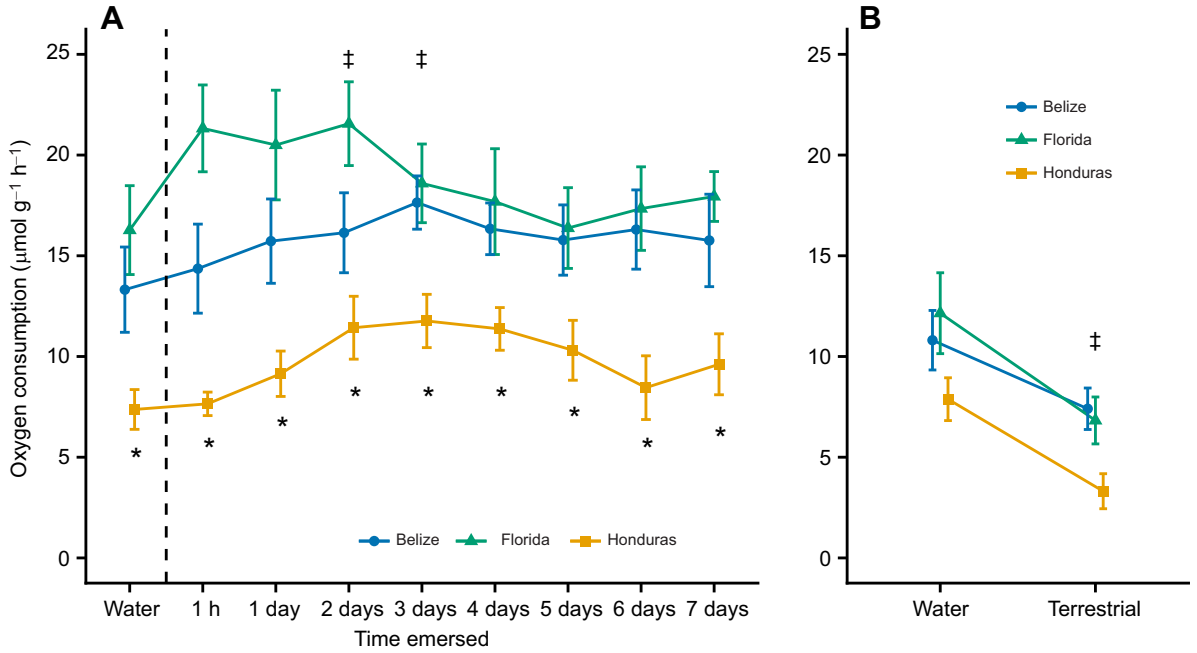

Fig. 2. Rates of O_2 consumption in the three strains of *K. marmoratus* in water and in air. O_2 consumption was measured (A) daily over 7 days of terrestrial acclimation, and (B) after 21 days of terrestrial acclimation. Data are presented as means±s.e.m. Asterisks denote a significant overall difference between the Honduran strain versus the other two strains ($P<0.05$), and double-daggers indicate significant overall differences in metabolic rate compared with the value in water ($P<0.05$).

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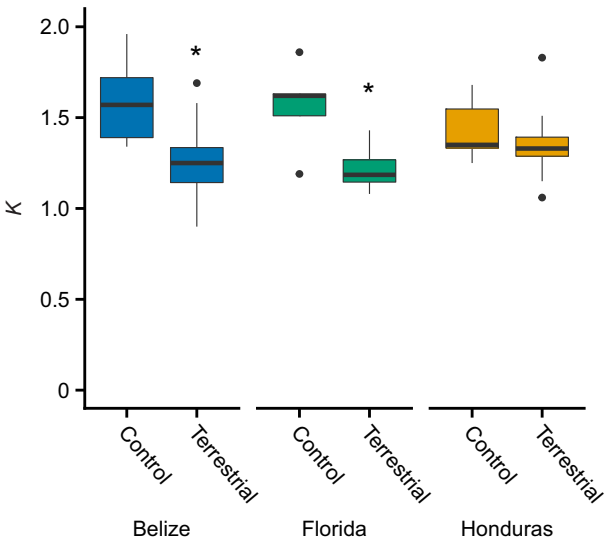

Fig. 3. Fulton's condition factor (*K*) in the three strains of *K. marmoratus* in water under normal conditions (control) and after 21 days out of water (terrestrial). For each boxplot, the bold horizontal line in the middle of the box represents the median, the top and bottom of the box represent the quartiles (i.e. 25th and 75th percentiles), whiskers show the highest and lowest values within 1.5× the interquartile range, and points show outliers. Asterisks represent significant differences within a strain (P<0.05).

strains (two-way ANOVA, $F_{1,110}$=1.4, P=0.23; Fig. 6F; Fig. S5C). Fish from control microcosms were longer (two-way ANOVA, $F_{1,110}$=168.04, P<0.001), heavier (two-way ANOVA, $F_{1,110}$=214.96, P<0.001), in better condition (two-way ANOVA, $F_{1,110}$=42.02, P<0.001) and had larger gonads (two-way ANOVA, $F_{1,110}$=7.77, P=0.006) than fish from fluctuating microcosms, but there was no difference in hepato-somatic index ($F_{1,110}$=3.70, P=0.057; Fig. 6; Fig. S5). After controlling for body length, total gill filament length was not different between strains (ANCOVA, $F_{1,82}$=1.22, P=0.27; Fig. S6) or microcosm conditions (ANCOVA, $F_{1,82}$=0.85, P=0.36; Fig. S6). Almost 11-fold more embryos were recovered from fluctuating (89.8±7.6) versus control (8.1±1.4) microcosms (Mann–Whitney, U=0, P<0.001). After accounting for the number of adult fish in each microcosm, embryo quantity was dependent on a significant strain-by-treatment interaction (ANCOVA, $F_{1,32}$=4.36, P=0.045). Honduras fish produced significantly more embryos than Belize fish under fluctuating conditions (Tukey, P=0.021; Fig. 7), but there was no difference between strains under control conditions (P=0.98). Two embryos collected from a single fluctuating microcosm were heterozygous at the microsatellite locus we used for identification. No adult males were found in the microcosm that contained the heterozygous embryos. Seven adult males were present at the end of the experiment, each in a different microcosm (five control, two fluctuating).

DISCUSSION

Using a self-fertilizing amphibious fish, we were able to directly relate survival out of water to genetically based metabolic and life-history phenotypes. In support of the proximate hypothesis that the overall pace of life determines the length of time non-aestivating *K. marmoratus* can survive out of water, we found that emersion tolerance was negatively associated with metabolic rate and the rate

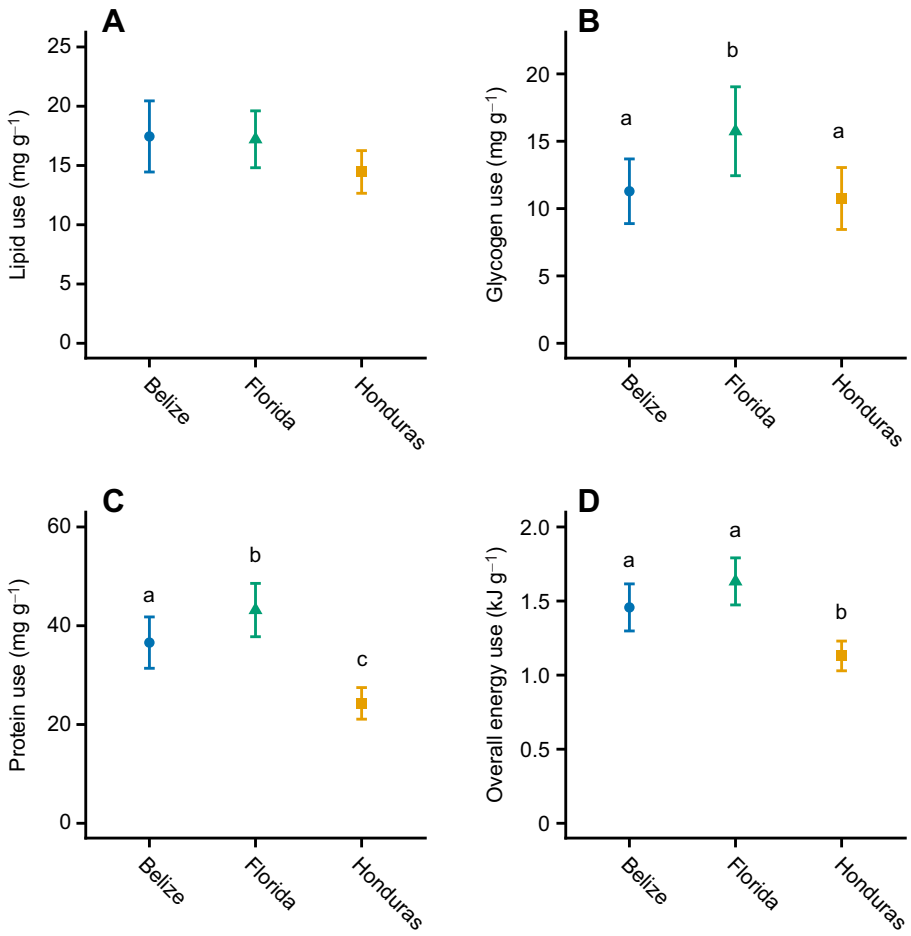

Fig. 4. Fuel use after 21 days of terrestrial acclimation in the three strains of *K. marmoratus*. (A) Lipid use, (B) glycogen use, (C) protein use and (D) total energy consumed. Data are presented as means±s.e.m. relative to wet mass. Different letters represent significant differences (P<0.05) between strains.

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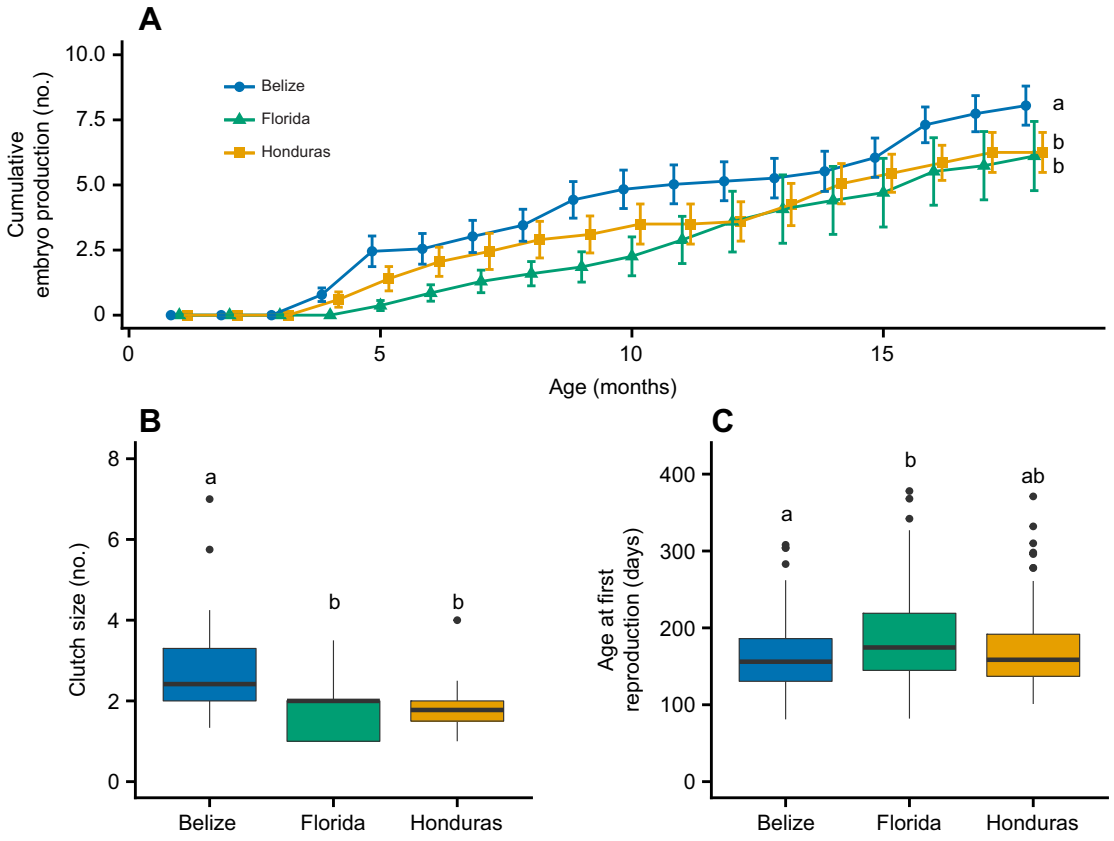

Fig. 5. Reproductive measures of the three strains of *K. marmoratus* over the first 18 months of life. (A) Total embryo production, (B) mean clutch size and (C) age at first reproduction. For each boxplot, the bold horizontal line in the middle of the box represents the median, the top and bottom of the box represent the quartiles (i.e. 25th and 75th percentiles), whiskers show the highest and lowest values within 1.5× the interquartile range, and points show outliers. Different letters represent significant differences (P<0.05) between strains.

of fuel use, but not the size of initial energy reserves. The emersion-tolerant Honduras strain of *K. marmoratus* also produced fewer offspring under normal laboratory conditions, as a result of an increased age of first reproduction and smaller average clutch size, consistent with a slow pace of life. We used long-term microcosms to test the ultimate hypothesis that a sub-lethal trade-off between emersion tolerance and aquatic performance mediates the relative advantages of different paces of life. Contrary to the prediction made by this hypothesis, slow Honduras fish did not outcompete relatively fast Belize fish (in terms of adult population size) under conditions of low water availability. However, Honduras fish produced more embryos than Belize fish in the water-limited condition, in support of the trade-off hypothesis. Honduras fish also tended to be larger and in better condition regardless of water availability, suggesting an overall advantage to low metabolic rate under our experimental conditions.

Metabolism and emersion tolerance

The rate of O_2 consumption in Honduras fish was consistently ∼40% lower than that of the other strains in both water and air, suggesting that this strain has an inherently slow metabolism that is genetically based. This difference was highly statistically significant in our 7 day terrestrial acclimation experiment (P<0.001), but was marginally non-significant (P=0.11) in our 21 day experiment despite being of similar magnitude (∼40%). Furthermore, the scope of metabolic depression after 21 days in air in Honduras fish was larger (58% reduction) than that of either the Belize (31% reduction) or Florida (44% reduction) strains, which probably allowed Honduras fish to conserve protein stores. Previous studies have suggested that *K. marmoratus* maintain or increase metabolic rate for several days after moving from water to land, but those experiments did not examine fish that were out of water for longer than 7 days (Ong et al., 2007; Blanchard et al., 2019). While probably helpful for survival during prolonged emersion, the scope of metabolic depression we found in *K. marmoratus* is smaller than has been measured in classically aestivating amphibious fishes such as *P. aethiopicus*, *Synbranchus marmoratus* and *Lepidogalaxias salamandroides*, which reduce O_2 consumption by 65–80% during months-long aestivation in mud (Guppy and Withers, 1999). Interestingly, the control rate of O_2 consumption in Honduras fish was almost identical to the depressed rate in the other two strains. One possibility is that the constitutively low metabolic rate of the Honduras strain evolved via the genetic assimilation of metabolic plasticity, which would be expected if these fish were found in habitats that regularly dried (Pigliucci et al., 2006; Lande, 2009). More research is required to understand the mechanism by which Honduras fish achieve low metabolic rate (e.g. increased efficiency or reduced expenditure), especially given the high growth rate of this strain. Overall, however, constitutive expression of a low baseline metabolic rate, combined with the ability to further reduce metabolism during extended periods without water, probably allows the Honduras strain to prolong survival out of water by conserving energy reserves.

There was a small but consistent increase in O_2 consumption after 2–3 days out of water across all three strains we investigated, even though fish remained largely motionless. Consistent with this

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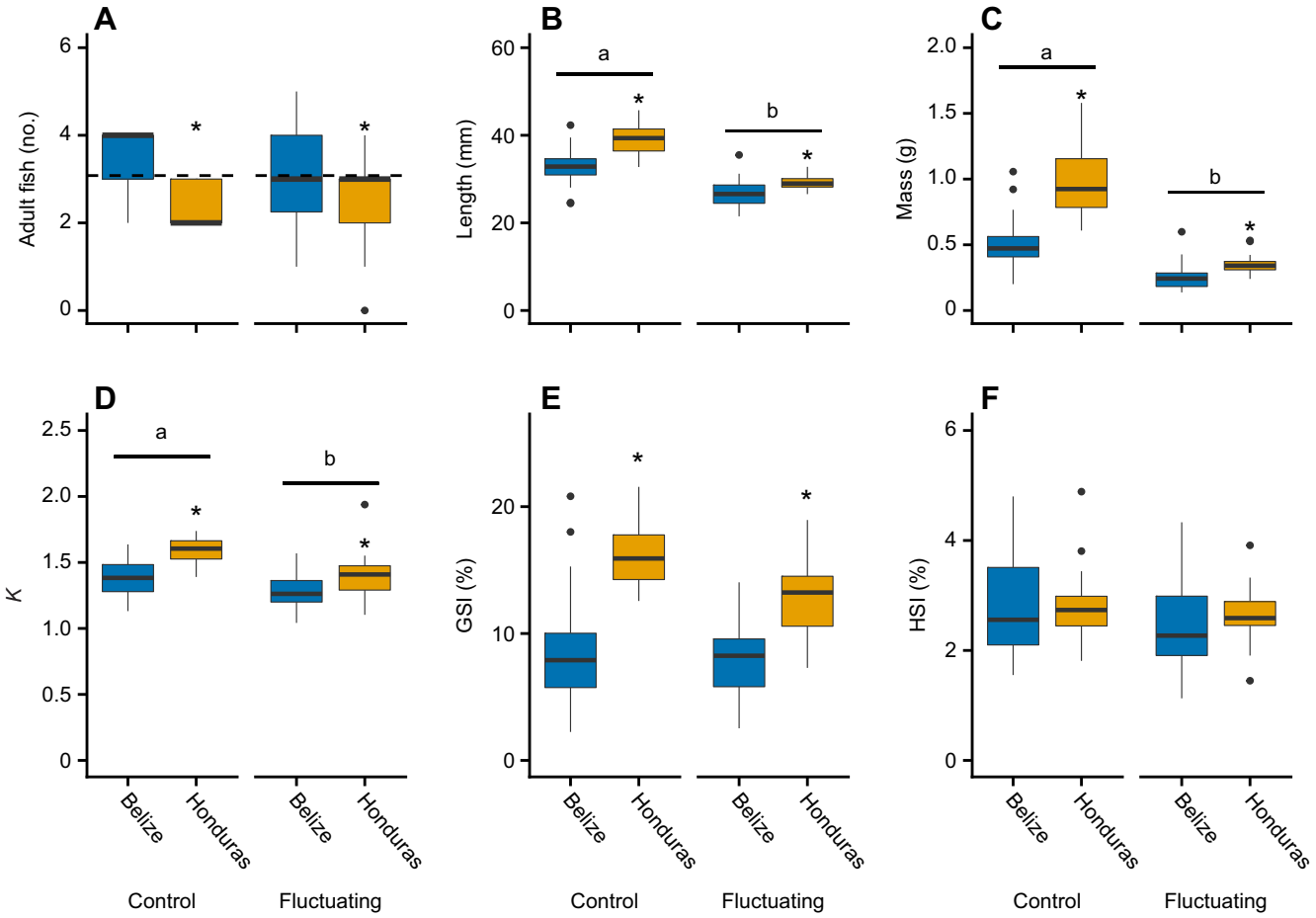

Fig. 6. Number, body size and condition of *K. marmoratus* after 12 months in fully aquatic (control) or periodically drained (fluctuating) microcosms. (A) Number of adult fish collected, (B) standard length, (C) wet mass, (D) Fulton's condition factor, (E) gonado-somatic index (GSI) and (F) hepato-somatic index (HSI). The dashed line in A indicates the number of fish of each strain initially placed in each microcosm. For each boxplot, the bold horizontal line in the middle of the box represents the median, the top and bottom of the box represent the quartiles (i.e. 25th and 75th percentiles), whiskers show the highest and lowest values within 1.5× the interquartile range, and points show outliers. Different letters represent a significant overall difference between treatment conditions, and asterisks denote a significant overall difference between strains (P<0.05).

finding, previous work found increased rates of CO_2 excretion in *K. marmoratus* over 5 days of terrestrial acclimation (Ong et al., 2007). This transient increase in metabolic rate may reflect the energetic cost of mounting phenotypically flexible responses during air exposure, such as gill remodelling (Ong et al., 2007), cutaneous angiogenesis (Cooper et al., 2012; Blanchard et al., 2019) and enlargement of cutaneous ionocytes (LeBlanc et al., 2010).

The pattern of energy use varied among strains in a manner consistent with the differences in whole-animal O_2 consumption. In independent experiments, Honduras fish lost the least amount of body mass relative to the other two strains and showed no change in condition factor after 21 days out of water. Protein catabolism was also lowest in Honduras fish. Generally, teleosts use protein and some lipids as fuel sources when food is not restricted, but glycogen and lipids are more important during starvation (Jobling, 1994; Moyes and West, 1995). However, African lungfish defend glycogen stores during aestivation, perhaps to facilitate rapid recovery when routine metabolism must be restored (Frick et al., 2008). After 21 days of emersion, all three strains of mangrove rivulus we tested had consumed a large fraction of their glycogen (~80% of initial) and lipid (~61%) stores, but only some protein (17–29%, depending on strain). This pattern resembles that of a typical starving teleost, rather than an aestivating lungfish. Presumably, the Florida and Belize fish, with their higher metabolic rates, began to metabolize protein earlier in the emersion period than the Honduras fish, resulting in greater consumption at the 21 day time point. In Honduras fish, larger protein reserves after 21 days of air exposure, in addition to fuelling continued emersion, may also help preserve locomotor ability when these fish return to water, similar to inactive hibernating mammals that protect protein stores to minimize impairment of skeletal muscle function when they emerge from winter dens (e.g. Hindle et al., 2015).

There were no differences in initial energy stores among the Belize, Florida and Honduras strains. One possible explanation is that there may be costs to carrying large energy stores, such as increased attractiveness to predators (Jensen et al., 2012) or decreased locomotory performance (Gibb et al., 2013). Alternatively, fish in the wild may detect environmental cues that indicate the onset of the dry season and respond by increasing internal energy stores (Griffiths and Kirkwood, 1995; Schultz and Conover, 1997). In our laboratory setting, however, such anticipatory feeding would not have been possible as the fish were given no signals that emersion was imminent.

Emersion tolerance trade-offs

Pace of life theory makes conflicting predictions about the direction of correlations between metabolic rate, growth and reproductive investment (Burton et al., 2011). According to the acquisition

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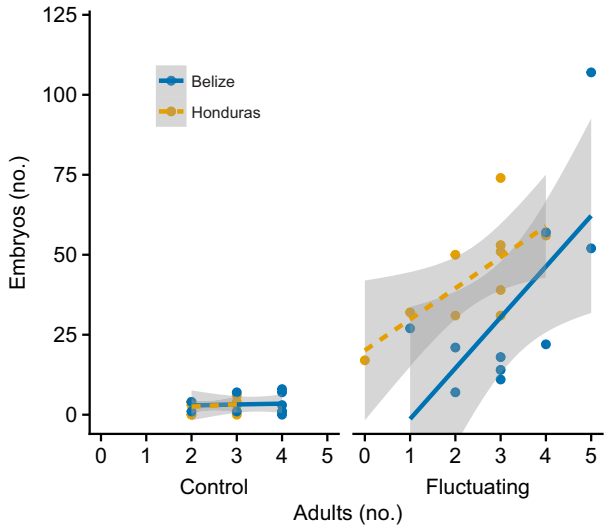

Fig. 7. Number of embryos recovered from microcosms after 12 months. More embryos were released by the Belize versus Honduras strain under fluctuating conditions (P<0.05).

model, a fast metabolism allows more resources to be acquired, leading to faster growth and increased reproductive investment (Mathot and Dingemanse, 2015). Conversely, the allocation model suggests that limited resources are split between various physiological processes via trade-offs; negative relationships between each of metabolic rate, growth and reproduction are thus expected (Burton et al., 2011). We found that Honduras fish, with the lowest metabolic rate, produced fewer embryos but grew faster than Belize fish under standard laboratory rearing conditions, suggesting a trade-off consistent with the allocation model. Furthermore, Honduras fish were much larger than Belize fish in both microcosm conditions. Although faster growth is typically correlated with high metabolic rates in animals (Allen et al., 2016), the negative relationship between growth and metabolic rate we found in *K. marmoratus* has also been found in some other fishes (Alvarez and Nicieza, 2005; Norin and Malte, 2011).

Our results support, in part, the hypothesis that differences in metabolic rate between mangrove rivulus strains are ultimately caused and maintained by a trade-off between emersion tolerance and aquatic performance. This hypothesis predicts that the slow Honduras fish would have higher fitness than Belize fish in fluctuating conditions when water was periodically unavailable, and this was indeed the case by several metrics. Honduras fish were larger, in better condition and produced more embryos than Belize fish in the fluctuating condition. However, Honduras fish were also larger in the control microcosms where there was no difference between strains in embryo production, in contrast to the prediction that Honduras fish would have lower fitness in constant aquatic conditions. Our results are consistent with many other studies of local adaptation, which often find that phenotypes that are advantageous under some conditions have neutral consequences in other environments, especially in highly heterogeneous environments (Bono et al., 2017).

It is not clear whether fast and slow phenotypes have effectively equal fitness under fully aquatic conditions, or whether there are situations that would favour the fast Belize phenotype. One limitation to our microcosm experiment was that fish were unable to disperse. The natural habitat of mangrove rivulus and other rivuline killifishes typically consists of a mosaic of small, intermittent pools (Turko and Wright, 2015; Furness et al., 2018; Sutton et al., 2018). A high metabolic rate is often correlated with high boldness and activity (e.g. Killen et al., 2011; Gangloff et al., 2017), and perhaps Belize fish are more likely to leave water and disperse to unoccupied habitats. We have previously found that metabolic rate of *K. marmoratus* was positively associated with the frequency of emersion behaviour (Turko et al., 2018). Embryo production by Belize fish was the highest of any strain in our laboratory colony, typical of a dispersal phenotype. Furthermore, when embryo production in the microcosm experiment was standardized to gonad mass, rather than the number of adults, the Belize strain produced almost twice as many embryos per gram of gonad compared with Honduras fish. However, we found that Belize fish had the lowest activity of any strain after forced air exposure, opposite to the prediction made by the dispersal phenotype hypothesis. We also found nearly 9-fold more embryos in fluctuating versus control conditions, despite the larger overall size of control fish. We think it is unlikely that this disparity reflects differences in reproductive output, but instead is the result of cannibalism under aquatic conditions (Wells et al., 2015). Our experiment ended when fluctuating microcosms had been without water for 1 week, so embryos deposited terrestrially during this period could not be consumed by the suction-feeding mangrove rivulus (Heiss et al., 2018), unlike embryos under control conditions. Presumably, most of the embryos produced under fluctuating conditions were cannibalized each time water returned, explaining the very low levels or recruitment we observed. Thus, Belize fish may live a traditionally fast pace of life that favours reproduction over growth, but the inability to escape competition/embryo cannibalism from the larger Honduras fish in our microcosms nullified the advantages of this life-history strategy. High metabolic rates are often correlated with aggression (Réale et al., 2010); thus, the fast Belize phenotype may be superior competitors in situations of relative food scarcity. Our conditions of *ad libitum* food provisioning would have failed to detect such a benefit to increased metabolic rate.

The androdioecious mating system of mangrove rivulus is thought to provide these fish with a mechanism to benefit from both self-fertilization and outcrossing, depending on the context (Ellison et al., 2011; Avise and Tatarenkov, 2015). This ‘best of both worlds’ hypothesis predicts that animals in relatively suitable habitats should self-fertilize to maximize the genetic inheritance by the offspring, while those in less suitable habitats should opt for outcrossing to increase the genetic variation of progeny so that some offspring will be better suited to the environmental conditions. In our experiments, this could be reflected as an increased number of male Belize fish and higher rate of outcrossing in fluctuating microcosms, while in control conditions more Honduras males and outcrossing would be predicted (as a result of environmental mismatching). We found no evidence consistent with these predictions. Only 7 of 114 adult fish were male, and 2 of 979 identified embryos were the result of outcrossing between strains. One possibility is that despite being without water for 6 out of 12 months, the fluctuating microcosms were not sufficiently stressful for the benefits of outcrossing to outweigh the cost of reduced genetic inheritance. Alternatively, the mating system of adult mangrove rivulus may not be responsive to environmental conditions and could instead rely on developmental plasticity or stochastic epigenetic effects that act on early life stages.

We found two embryos that were heterozygous at the microsatellite locus we studied, indicating that these resulted from outcrossing. The current view is that males are required for outcrossing (Avise and Tatarenkov, 2015; Furness et al., 2015), but no males were found in the microcosm that contained both of these outcrossed embryos. One possibility is that a male(s) was present when the embryos were fertilized, but subsequently died and decomposed before the microcosms were sampled. Alternatively,

these embryos resulted from outcrossing between a hermaphroditic individual of each strain. If so, these two embryos would represent a hermaphrodite-driven outcrossing rate of ∼0.2% given that we genotyped 831 embryos, consistent with the lack of outcrossing reported by Furness et al. (2015) in a sample of 173 embryos.

Conclusions and perspective

Our findings show that pace of life was associated with emersion tolerance in *K. marmoratus* out of water, which allowed the slow Honduras strain to survive out of water for an average of 12 days (∼21%) longer than the relatively fast Belize strain. Furthermore, Honduras fish had a lower metabolic rate under control aquatic conditions, indicating genetic divergence between strains that could possibly be the result of genetic assimilation of metabolic plasticity (Pigliucci et al., 2006; Lande, 2009). These results are consistent with other work showing generally slow lifestyles in extremophile fishes (Passow et al., 2017). We did not detect an obvious cost to emersion tolerance in our microcosm experiment, consistent with other studies of local adaptation that have often found conditional neutrality of variable traits instead of antagonistic trade-offs (Bono et al., 2017). We also did not push environmental extremes to the limit, as we were interested in investigating only sub-lethal effects, and life-history trade-offs are often only revealed under extreme environmental conditions (Lemaître et al., 2015).

Recently, we discovered genetically divergent wild populations of *K. marmoratus* occupying abiotically distinct habitats (high versus low water availability), and the phenotypes of these populations matched those predicted by pace of life theory (Turko et al., 2018). The population that inhabits an ephemeral pond (no water during the dry season) tended to be larger, in better body condition, and had metabolic rates that were ∼30% lower than those of fish from a nearby site with higher water availability. Together with our laboratory data, these findings support the idea that environmental heterogeneity is an important factor that drives differences in metabolic rate and pace of life.

Acknowledgements

We thank Nick Bernier, Doug Fudge, Todd Gillis and Graham Scott for helpful discussions regarding experimental design, Chunfang Wang, Patricio Saez and Dominique Bureau for assistance measuring protein content, and Matt Cornish, Mike Davies and Bianca Cisternino for animal care.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.J.T., J.E.D., K.L., R.L.E., P.A.W.; Methodology: A.J.T., J.E.D., P.K., R.L.E., P.A.W.; Formal analysis: A.J.T.; Investigation: A.J.T., J.E.D., I.Y.-L., K.L., P.K., J.M.H., R.L.E.; Resources: R.L.E., P.A.W.; Data curation: A.J.T.; Writing - original draft: A.J.T.; Writing - review & editing: J.E.D., I.Y.-L., P.K., R.L.E., P.A.W.; Visualization: A.J.T.; Supervision: R.L.E., P.A.W.; Project administration: A.J.T., R.L.E., P.A.W.; Funding acquisition: P.A.W.

Funding

Funding was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) Graduate Scholarship to A.J.T. and an NSERC Discovery grant to P.A.W.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.209270.supplemental

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