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RESEARCH ARTICLE

Fish embryos on land: terrestrial embryo deposition lowers oxygen uptake without altering growth or survival in the amphibious fish *Kryptolebias marmoratus*

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ABSTRACT

Few teleost fishes incubate embryos out of water, but the oxygen-rich terrestrial environment could provide advantages for early growth and development. We tested the hypothesis that embryonic oxygen uptake is limited in aquatic environments relative to air using the self-fertilizing amphibious mangrove rivulus, *Kryptolebias marmoratus*, which typically inhabits hypoxic, water-filled crab burrows. We found that adult mangrove rivulus released twice as many embryos in terrestrial versus aquatic environments and that air-reared embryos had accelerated developmental rates. Surprisingly, air-reared embryos consumed 44% less oxygen and possessed larger yolk reserves, but attained the same mass, length and chorion thickness. Water-reared embryos moved their opercula ∼2.5 more times per minute compared with air-reared embryos at 7 days post-release, which probably contributed to the higher rates of oxygen uptake and yolk utilization we observed. Genetically identical air- and water-reared embryos from the same parent were raised to maturity, but the embryonic environment did not affect growth, reproduction or emersion ability in adults. Therefore, although aspects of early development were plastic, these early differences were not sustained into adulthood. *Kryptolebias marmoratus* embryos hatched out of water when exposed to aerial hypoxia. We conclude that exposure to a terrestrial environment reduces the energetic costs of development partly by reducing the necessity of embryonic movements to dispel stagnant boundary layers. Terrestrial incubation of young would be especially beneficial to amphibious fishes that occupy aquatic habitats of poor water quality, assuming low terrestrial predation and desiccation risks.

KEY WORDS: Developmental plasticity, Developmental rate, Oxygen consumption, Operculum, Chorion, Mangrove rivulus

INTRODUCTION

Oxygen availability significantly impacts the early development and growth of fishes because embryos are generally considered to be oxygen conformers (e.g. Matschak et al., 1997; Barrionuevo and Burggren, 1999; Miller et al., 2008). Hypoxia delays development in fishes (e.g. Alderdice et al., 1958; Garside, 1959, 1966; Silver et al., 1963; Miller et al., 2011; Bianchini and Wright, 2013; Robertson et al., 2014). In contrast, embryos of amphibious fishes may experience relatively high oxygen availability if deposited out of water because the diffusion coefficient of oxygen in air is ∼8000 times higher than in water, and boundary layers in air are also much smaller (Dejours, 1988).

Terrestrial development allows developing embryos to exploit abundant oxygen, but also exposes embryos to the risk of desiccation (Marco, 2001; Touchon and Warkentin, 2010; Touchon and Worley, 2015). To survive in a terrestrial environment, fish embryos are typically deposited in moist environments (Frank and Leggett, 1981; Middaugh, 1981; Taylor, 1999; Martin and Swiderski, 2001; McDowall and Charteris, 2006; Martin, 2015). For example, California grunion (*Leuresthes tenuis*) spawn in damp sand, which offers embryos physical protection and higher oxygen levels relative to water (Walker, 1949; Darken et al. 1998; Martin et al., 2011). The Japanese mudskipper *Periophthalmus modestus* incubates embryos in air-filled chambers under water, built into mudflats and filled with air by the action of the parents (Ishimatsu et al., 2007). Mummichog (*Fundulus heteroclitus*) embryos are periodically exposed to an aerial environment during low tides where they hatch earlier relative to embryos in water (Taylor et al., 1977; Tingaud-Sequeira et al., 2009, 2013). Finally, embryos of some other killifish species, such as *Austrofundulus limnaeus*, enter diapause under terrestrial conditions and limit desiccation by reducing water permeability (Podrabsky et al., 2001). However, to our knowledge there have been no studies comparing the metabolic trade-offs of development in fully terrestrial or fully aquatic environments.

There is some evidence that oxygen regulation occurs in amphibians that incubate their embryos terrestrially [e.g. African clawed frog *Xenopus laevis* (Hastings and Burggren, 1995); tropical frog *Eleutherodactylus coqui* (Burggren et al., 1990)]. Terrestrial red-eyed treefrog (*Agalychnis callidryas*) embryos move their bodies to reduce boundary layers and mix perivitelline fluid, thereby increasing oxygen availability and supporting a higher metabolic rate (Warkentin et al., 2005). Embryonic movement of fully aquatic rainbow trout (*Oncorhynchus mykiss*) embryos also increased prior to hatching, possibly as a strategy to enhance oxygen transport by mixing the perivitelline fluid within the chorion to dispel boundary layers (Ninness et al., 2006). Thus, although there is evidence that embryos are typically oxygen conformers, there may be environmental situations where embryos regulate oxygen diffusion by increasing movement within the chorion.

The amphibious mangrove rivulus, *Kryptolebias marmoratus* (Poey), is an excellent model species in which to address questions related to early development in aquatic and terrestrial environments (Mourabit et al., 2011). Adult *K. marmoratus* often leave the water, and adults have been observed to release embryos in both aquatic and terrestrial habitats in the laboratory (M.W.W., unpublished observation). While information on the natural spawning locations of *K. marmoratus* is scarce, embryos have been observed in terrestrial environments near crab burrows, the primary aquatic

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habitat of adults (Taylor, 2012). Furthermore, mangrove rivulus possess a unique reproductive system, internal self-fertilization, which allows homozygous adult hermaphrodites to produce isogenic offspring (Harrington, 1961). The unique reproductive system of adults makes them an excellent model for studying how the environment affects development, as genetic differences can be eliminated (Turko et al., 2011; Earley et al., 2012).

We hypothesized that embryonic oxygen uptake is limited in aquatic relative to aerial environments. We predicted that if the terrestrial environment provides greater oxygen availability for embryonic *K. marmoratus*, adults should release more embryos terrestrially. Furthermore, embryonic development and metabolism should be enhanced in terrestrial relative to aquatic environments and embryos should show behaviours that enhance oxygen exchange in water to minimize boundary layers within the chorion. We then tested whether different external oxygen concentrations in both terrestrial and aquatic environments would trigger hatching. Finally, we tested whether faster growth rates or larger energy reserves resulting from terrestrial development would translate into adult fish with an altered phenotype (i.e. developmental plasticity) (West-Eberhard, 2003). We predicted that the higher oxygen availability that embryos experience in an early terrestrial environment would result in increased adult body size, decreased age at first reproduction, increased reproductive output, and increased emersion tolerance compared to adults reared in water as embryos.

RESULTS

Series I: reproductive output of adult *K. marmoratus* in water or air

Adult *K. marmoratus* exposed to a terrestrial environment released more than twice as many embryos after 96 h as in water (0.724±0.15 embryos/adult in air, 0.276±0.10 embryos/adult in water; t=2.50, d.f.=57, P=0.015, N=58). In terrestrial conditions, 38% of adults released embryos compared with 15% in water.

Series II: impact of rearing environment on early development and metabolism

Embryos exposed to air or water at 7 days post-release (dpr) were at the same developmental stage (stage 28; Fig. S1). At 15 dpr, however, a greater number of air-reared embryos had reached hatching competency (95% at stage 32) relative to those reared in water (45% at stage 32; Fig. S1). By 30 dpr, all embryos had reached the final stage of development (100% at stage 32; Fig. S1).

The mean standard length and body depth of *K. marmoratus* embryos were not significantly different between treatments (body depth t=1.49, d.f.=22.5, P=0.15; standard length t=−0.59, d.f.=22.9, P=0.56; Table 1). Similarly, the thickness of the chorion was not significantly different between embryos reared in air or water at 30 dpr (t=1.441, d.f.=18, P=0.167; Table 1). In both water- and air-reared embryos, there were five distinct ‘lamellae’ in the inner chorionic layer that stained positively for collagen (Fig. S2).

Table 1. Parameters for *Kryptolebias marmoratus* embryos reared in fully aquatic or moist terrestrial environments at 30 dpr

Parameter	Water-reared	Air-reared
Standard length (mm)	4.46±0.09	4.38±0.10
Body depth (mm)	0.745±0.022	0.796±0.026
Yolk sac surface area (mm^2)	0.125±0.029	0.224±0.035*
Chorion thickness (µm)	8.09±0.51	9.01±0.39

Data are means±s.e. (N=12). dpr, days post-release.
*Significant difference (P<0.05).

Embryos reared out of water had a yolk sac surface area 2-fold higher relative to that of aquatic animals at 30 dpr (t=2.21, d.f.=22.6, P=0.037; Table 1). The wet mass of the embryo with and without the chorion, dry mass of the embryo and body water content were not affected by rearing environment (Table 2). Embryonic wet and dry mass increased with development as expected (P<0.001; Table 2).

Overall, air-reared embryos consumed ∼44% less oxygen than embryos reared in water (F=40.8, d.f.=1,34, P<0.001; Fig. 1A). Embryos reared in water showed a significantly different pattern of oxygen consumption over developmental time compared with air-reared embryos (interaction F=3.29, d.f.=2,34, P=0.049). In water, oxygen consumption significantly decreased at 30 dpr relative to 15 dpr (F=6.10, d.f.=2,34, P=0.0054; Fig. 1A). However, oxygen consumption in air-reared embryos was consistent between 15 and 30 dpr (Fig. 1A). The aquatic oxygen consumption of air-reared embryos that were subsequently returned to water was 2-fold higher after 1 h than that of embryos reared in water and tested in water (t=4.07, d.f.=7.84, P=0.0037; Fig. 1B). Lactate concentrations were not different between air- and water-reared embryos at 30 dpr (water-reared=0.42±0.04 µmol embryo^{-1}; air-reared=0.50±0.05 µmol embryo^{-1}; t=1.22, d.f.=18, P=0.24).

Series III: impact of rearing environment on embryo movement

Air-reared embryos showed significantly fewer (∼2.5-fold) opercular movements (9.74±0.32 min^{-1}) at 7 dpr compared with embryos reared in water (23.11±2.61 min^{-1}; t=5.929, P<0.001; Fig. 2), but this difference disappeared at 15 and 30 dpr (Fig. 2, Movie 1). Opercular movements generally decreased over development (7 versus 15 dpr t=4.865, P<0.001; 15 versus 30 dpr t=3.737, P<0.001; Fig. 2). The number of large body rotations within the chorion was unaffected by the age of the embryo and the environment in which it was reared (water reared 0.42±0.06 movements h^{-1}, air reared 0.43±0.10 movements h^{-1}).

Air-reared embryos that were subsequently returned to water at 30 dpr had a significantly higher number of opercular movements after 1 h compared with individuals of the same age that were reared in water (t=3.75, d.f.=6.09, P=0.0092; Fig. 2). There was a significant positive relationship between oxygen consumption and the number of opercular movements per minute in embryos regardless of rearing environment (R^2=0.8857, F=38.7, d.f.=1,6, P=0.002; Fig. 3).

Series IV: environmental hatching triggers

Water-reared embryos (30 dpr) did not hatch when exposed to normoxic brackish water (control conditions), normoxic freshwater or normoxic air after 1 h (Fig. 4). Acute exposure to hypoxic water [10% dissolved oxygen (DO) saturation] triggered hatching in 24.1±2.3 min and all embryos hatched within 35 min. Embryos exposed to hypoxic air (10% DO saturation) hatched significantly more slowly than those exposed to aquatic hypoxia (t=2.170, d.f. =13, P=0.049), requiring 37.5±5.7 min of hypoxic exposure. All but one of the embryos in the hypoxic air group (7 of 8 embryos) hatched within the 1 h exposure period; the unhatched embryo hatched shortly after being returned to control water conditions.

Series V: long-term effects of rearing environment

Exposure to a terrestrial or aquatic environment during early development did not significantly affect the mass or length of adult fish (Table 3). Similarly, neither fecundity nor the age at first reproduction were affected by the environmental conditions fish

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Table 2. Mass of embryos reared in air or water for 7, 15 or 30 dpr

Treatment	7 dpr	15 dpr	30 dpr
Air			
Wet mass, with chorion (mg)	1.81±0.07	1.95±0.06	1.83±0.15
Wet mass, embryo only (mg)	0.36±0.02^{a}	0.73±0.05^{b}	0.76±0.02^{c}
Dry mass, embryo only (mg)	0.15±0.04^{a}	0.16±0.03^{a,b}	0.19±0.02^{b}
Body water content (%)	73.3±3.0	77.1±4.1	75.2±1.7
Water			
Wet mass, with chorion (mg)	1.70±0.06	1.76±0.12	1.83±0.07
Wet mass, embryo only (mg)	0.48±0.04^{a}	0.65±0.04^{b}	0.86±0.04^{c}
Dry mass, embryo only (mg)	0.12±0.02^{a}	0.17±0.02^{b}	0.22±0.03^{c}
Body water content (%)	76.7±4.6	73.1±1.9	74.0±3.6

Data are means±s.e. (*N*=8–10).
Wet/dry mass of the embryo only is given as the wet/dry mass of the dechorionated and deyolked embryo.
Superscript lowercase letters indicate significant differences between developmental stages.

experienced as embryos (Table 3). Adult emersion tolerance was also unaffected by the embryonic environment (Fig. S3). The mean survival in air of adults reared in water as embryos was 37.2±1.9 days, whereas in adults air-reared as embryos it was 36.8±2.7 days.

DISCUSSION

This is the first time that the spawning rates of an amphibious fish have been measured in both terrestrial and aquatic environments. We demonstrate that *K. marmoratus* released two times more embryos out of water and these embryos have an altered physiological trajectory relative to embryos reared in water, but reach the same end point. Terrestrial incubation accelerated early development and substantially decreased yolk utilization compared with embryos in water. Greater yolk reserves at hatching competence in embryos out of water would allow for a delay in hatching (Moravek and Martin, 2011). The metabolic rate of *K. marmoratus* embryos reared in air was ∼44% lower than that of embryos reared in water, indicating differences in energetic costs depending on rearing environment. Embryos reared in water had increased opercular movements in early ontogeny compared with air-reared embryos, partly explaining the higher metabolic rate. When air-reared embryos were acutely returned to water, they increased movement and metabolic rate, supporting the hypothesis that *K. marmoratus* embryos use micro-environment manipulation to maintain oxygen uptake or initiate a hatching response. Indeed, there was a very tight correlation between opercular movements and oxygen uptake (R^2=0.9) across all embryo groups. Adults followed through to maturity were not significantly different in body size or reproductive output, nor were they better at surviving a subsequent terrestrial episode. Taken together, our results show that terrestrial incubation is an effective strategy to avoid a variable and harsh aquatic environment with some early energetic benefits and no obvious long-term consequences.

We tested the hypothesis that embryonic oxygen uptake is limited in aquatic environments relative to development out of water. As predicted, adults released more embryos in air relative to water, suggesting a preference for terrestrial environments. The clutch size was relatively low, consistent with previous reports in *K. marmoratus* (0.7–2.2 embryos per week; Grageda et al., 2005). Previous literature described anecdotal observations of adult *K. marmoratus* that released embryos terrestrially in a terrarium (Abel et al., 1987; Taylor, 1990) or in the wild near crab burrows (Taylor, 2012). The greater number of embryos released in air suggests that although adults use both environments for the release of embryos, they may prefer a terrestrial environment for embryo deposition under certain circumstances. To show a true preference, however, fish should be tested in a chamber with an artificial habitat that contained both aquatic and terrestrial spawning sites.

The developmental rate and energetic costs were different between embryos reared in water and air; however, embryo morphology (e.g. standard length, body depth) and mass were not affected by rearing environment. In contrast, air-reared *F. heteroclitus* embryos attained a greater body mass and length relative to water-reared embryos (Tingaud-Sequeira et al., 2009). After 7 days of development, *K. marmoratus* embryos had reached the same stage regardless of rearing environment; however, by 15 dpr, embryos reared in air were hatching competent (stage 32) while embryos reared in water were not (stage 31). These results provide some evidence for the prediction that embryos develop at a faster rate in a terrestrial environment. Similarly, air-exposed *F. heteroclitus* embryos hatched earlier than water-exposed embryos when placed in seawater (Tingaud-Sequeira et al., 2009). The

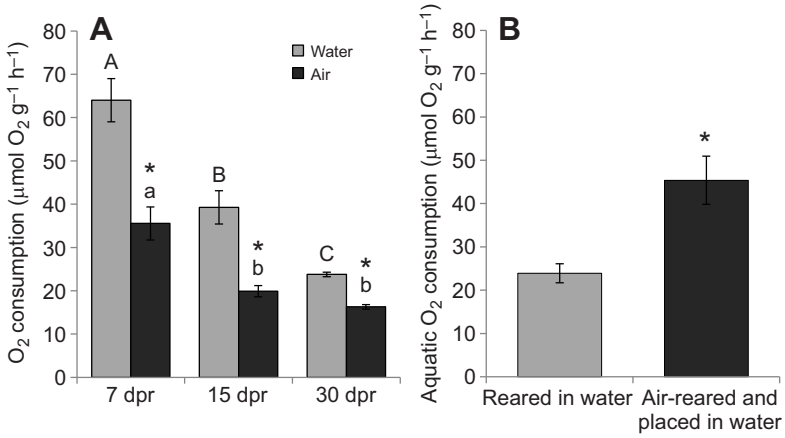

Fig. 1. Oxygen consumption of *Kryptolebias marmoratus* embryos. (A) Embryos were reared in water or air for 7, 15 and 30 days post-release (dpr) and measured in their respective respiratory media. Different uppercase letters indicate significant differences between developmental stages (7, 15 and 30 dpr) for embryos reared in water; different lowercase letters indicate significant differences between developmental stages for embryos reared in air. Asterisks indicate significant differences between embryonic rearing environments (P<0.05). (B) Embryos were reared in air or water for 30 days and placed in water for measurement. The asterisk indicates a significant difference between treatments (P<0.05). Data in A and B are presented as means±s.e.m. (*N*=6–9).

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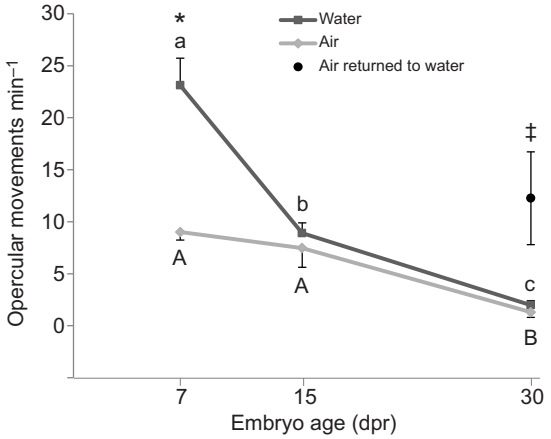

Fig. 2. Number of opercular movements per minute for *K. marmoratus* embryos in air or water at 7, 15 and 30 dpr. Data are presented as means±s.e.m. (*N*=8–10). Different lowercase letters indicate significant differences between stages (7, 15 and 30 dpr) for embryos reared in water; different uppercase letters indicate significant differences between embryonic stages for embryos reared in air. The asterisk indicates a significant difference between embryonic rearing environments and the double-dagger indicates a significant difference between air-reared embryos in air and air-reared embryos in water (P<0.05).

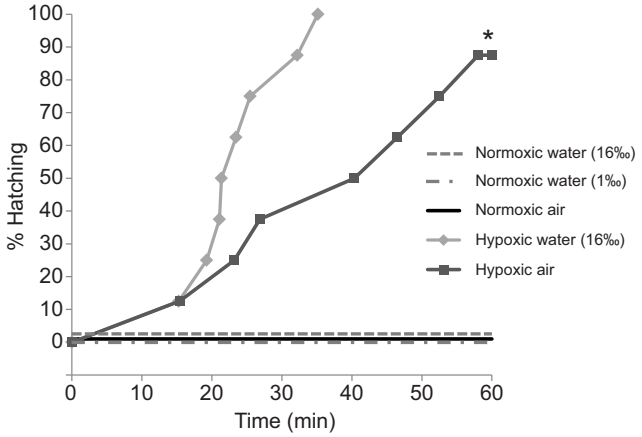

Fig. 4. Percentage of *K. marmoratus* embryos hatching at 30 dpr following acute exposure to normoxic brackish water, normoxic freshwater, hypoxic brackish water, normoxic air or hypoxic air. *N*=8. Normoxic brackish water, normoxic freshwater and normoxic air data were pooled as hatching was not observed over the 60 min. The asterisk indicates there was a significantly slower time to hatch for the hypoxic air relative to the hypoxic water group (t=2.170, d.f.=13, P=0.049).

natural hatching time of *K. marmoratus* is extremely variable (between 20 and 90 days; Sakakura and Noakes, 2000) and may depend on environmental stimuli, which may partly explain why air-reared embryos did not hatch at 15 dpr, despite being hatching competent. Typically, teleost embryos must be in water to trigger hatching and therefore the ability to delay hatching is helpful when they incubate partially or completely out of water (Brown and Green, 2014; Ishimatsu and Graham, 2011; Martin, 2015; Tingaud-Sequeira et al., 2009).

The metabolic rate of air-reared embryos was significantly lower than that of embryos reared in water at the three time points measured, in contrast to our prediction. Similarly, air-reared brown toadlet *Pseudophryne bibroni* embryos had lower metabolic rates close to hatch relative to water-reared embryos (Bradford and Seymour, 1985).

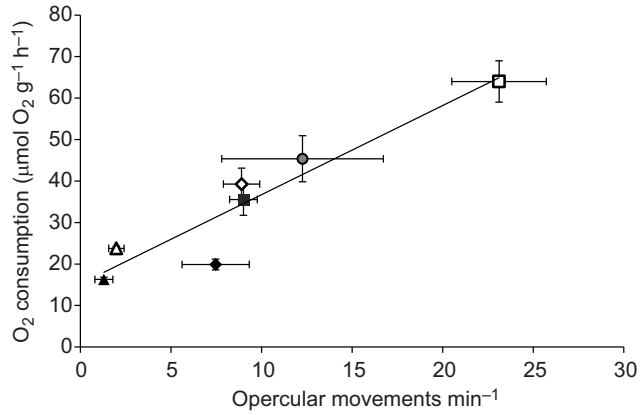

Fig. 3. Linear regression between oxygen consumption and opercular movements of embryos. There was a significant correlation between metabolic rate and the number of opercular movements per minute (P<0.05; y=2.1498x+15.21). Open symbols denote measurements in air, filled symbols denote measurements in water. Squares indicate embryos at 7 dpr, diamonds indicate embryos at 15 dpr, triangles indicate embryos at 30 dpr. The grey circle denotes embryos that were reared in air and returned to water at 30 dpr. Data are presented as means±s.e.m.

However, Bradford and Seymour (1985) did not observe significant differences in oxygen consumption at earlier developmental stages. We propose that the lower metabolic rate in the terrestrial environment is related in part to fewer opercular movements. In encapsulated fish embryos, relatively thick boundary layers develop in low flow environments that may impair oxygen transport in aquatic environments (Ciuhanda et al., 2007; Miller et al., 2008; Dhiyebi et al., 2013). We found evidence to support the hypothesis that embryos manipulate their micro-environment to maintain oxygen delivery. Water-reared embryos moved their opercula ∼3-fold more times than air-reared embryos at 7 dpr, possibly to dispel boundary layers and maintain oxygen uptake. Warkentin et al. (2005) also suggested that developing amphibians increased body movements within the chorion to help disrupt stagnant boundary layers. In developing embryos, energy is allocated to body maintenance, growth and maturation. By reducing the number of opercular movements required to maintain oxygen uptake early in development, air-reared embryos may have been able to allocate more energy to development and maturation than water-reared embryos, and thus increase their rate of development (Mueller et al., 2012). In contrast, the increased number of opercular movements and correlated increases in oxygen consumption in water-reared embryos probably required the use of a greater portion of available energy stores (yolk), which may at least partially explain the slower developmental rate up to 15 dpr and also the increased yolk sac utilization compared with air-reared embryos. Finally, there was no evidence that the lower oxygen uptake in air-exposed embryos was related to anaerobiosis, as lactate levels were similar between the two treatment groups.

The correlation between opercular movements and oxygen uptake was close to 1. Using the regression equation, we calculated that each opercular movement uses between 0.0167 nmol of oxygen at 7 dpr and 0.0283 nmol of oxygen at 30 dpr. When air-reared embryos (30 dpr) were acutely returned to water and increased opercular movement 7-fold, they consumed 0.032 µmol O_2 h^{-1} or 64% more than embryos not ventilating. There are few studies that have directly measured the metabolic cost of ventilation, but in adult rainbow trout (*Oncorhynchus mykiss*), switching from active ventilation to ram ventilation decreased oxygen consumption by ∼10% (Steffensen, 1985). The cost of opercular ventilation appears to be much higher in

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Table 3. Parameters for adult *K. marmoratus* reared as embryos in either aquatic or terrestrial environments

Parameter	Water-reared as embryos	Air-reared as embryos
Mass (g)	0.075±0.002	0.075±0.002
Standard length (mm)	19.4±0.3	19.2±0.2
Fecundity (embryos week^{-1})	0.29±0.07	0.42±0.1
Age at first reproduction (weeks)	23.8±0.7	28.6±1.9

Data are means±s.e. (water-reared *N*=24, air-reared *N*=22).
Mass and standard length were measured at 10 months of age.

early life stages compared with that in adults, probably because of the small size of the operculum in embryos and proportionally higher costs of overcoming viscous forces (Vogel, 1981).

The increased oxygen consumption of embryos returned to water may also be related to the initiation of a hatching response (DiMichele and Taylor, 1981). We found that *K. marmoratus* embryos hatch when acutely (~1 h) exposed to hypoxia in both terrestrial and aquatic environments, although normoxic air exposure was not a trigger. Aquatic hypoxia is a known trigger in several killifish and amphibians (DiMichele and Taylor, 1980; Levels et al., 1986; Bradford and Seymour, 1988), but this is the first report in *K. marmoratus*. Because of the differences in oxygen diffusion between media, even well-oxygenated water has limited oxygen available compared with air, which may have induced a hatching response in air-reared *K. marmoratus* embryos returned to water. When exposed to an environmental hatching trigger, California grunion increase oxygen consumption and the number of movements within the chorion (Speer-Blank and Martin, 2004; Martin et al., 2011). Besides the mechanical energy required for hatching, energy is also required for chemical digestion of the chorion by chorionase (Korwin-Kossakowski, 2012). Thus, it is possible that the observed increase in oxygen consumption and embryonic movements when air-reared embryos were returned to water were caused by the initiation of a hatching response; however, a longer measurement period would be necessary to test this hypothesis.

Fish embryos out of water may benefit from the increased availability of oxygen, but must avoid desiccation stress. One strategy might be to thicken or otherwise modify the chorion to prevent loss of water; however we found no evidence for this hypothesis. There were no differences in chorion structure between embryos reared in water and air. Furthermore, the chorion of *K. marmoratus* embryos (8–9 µm thick) was comparable to those of similarly sized fishes such as *F. heteroclitus* (13 µm; Shanklin, 1959) and *Oryzias latipes* (12–15 µm; Hart et al., 1984), but thicker than that of *Danio rerio* (0.5–0.6 µm; Joo and Kim, 2013). Also similar to most other fishes, the inner layer of the *K. marmoratus* chorion was composed of five fibrous 'lamellae' (Laale, 1980). These lamellae stained positively for collagen, a component of the extracellular matrix not commonly described in the chorion. Generally, the chorion surrounding fish embryos out of water is probably more important for providing mechanical protection than preventing water loss, as even in the severely water-challenged annual killifish *A. limnaeus* the chorion does not appear to play a role in water conservation (Podrabsky et al., 2001).

Our results suggest that there are no obvious long-term negative consequences of development in terrestrial environments compared with aquatic environments. Many studies have shown, in a variety of species, that altered environments during critical periods of early life stages permanently change the adult phenotype (West-Eberhard, 2003). However, in the present study, adults that were exposed to either an aquatic or a terrestrial environment in early development had similar body morphology, fecundity, age at first reproduction and survival in a subsequent terrestrial challenge. It is interesting that *K. marmoratus* embryos are able to adjust development in an aerial environment yet have no lasting consequences. This suggests that development has become relatively canalized (Waddington, 1942). Such a strategy may allow mangrove rivulus to spawn year round and exploit the best available aquatic or terrestrial niches without compromising the fitness of their offspring.

In summary, we observed that *K. marmoratus* deposit embryos in both aquatic and terrestrial artificial environments. Terrestrial incubation increased developmental rates while reducing the energetic costs of development. This partially supports our hypothesis that adult *K. marmoratus* deposit embryos out of water as a strategy to exploit the greater oxygen available in air. The decreased energetic cost of terrestrial development may have partly resulted from reduced movement. Opercular movements, which help to circulate perivitelline fluids within the chorion, were highly correlated with O_2 uptake. A decreased metabolic cost of development would be advantageous if an embryo needs to delay hatching until environmental conditions are suitable. Larger yolk reserves could also be advantageous if hatchlings initially enter a habitat with limited food resources. Development out of water had no long-term consequences on the adult phenotype, demonstrating canalization of embryonic development in *K. marmoratus*.

MATERIALS AND METHODS

Animal care

Kryptolebias marmoratus were maintained at the Hagen Aqualab, University of Guelph, ON, Canada, under standard laboratory conditions (25°C, 16‰, pH 8, 12 h light:12 h dark photoperiod; Frick and Wright, 2002) and originated from Twin Cayes, Belize (50.91 strain; Tatarenkov et al., 2010). All experiments were approved by the University of Guelph Animal Care Committee.

Series I: reproductive output of adult *K. marmoratus* in water or air

To measure the fecundity of *K. marmoratus* in air and water, sexually mature adults (>5 months old, *N*=58) were placed in individual 100 ml containers and monitored for 96 h. Fish were exposed to both terrestrial and aquatic environments in a randomly determined order 1 week apart for pair-wise comparisons. For the water treatment, adults were placed individually in 100 ml plastic specimen containers with 50 ml of brackish water (25°C, 16‰, pH 8). In water, the addition of a small (5 cm diameter), inverted filter paper funnel (Whatman, GE Healthcare Companies, Little Chalfont, UK) elevated off the bottom of the plastic container allowed embryos to fall out of reach of the parent to prevent any possible cannibalism. In air, fish were placed on a piece of moist filter paper in contact with a moist reservoir (three cotton balls soaked with water; 25°C, 16‰, pH 8, >99% relative humidity; Ong et al., 2007) in 100 ml containers. Consumption of embryos was not explicitly prevented during air exposure, but preliminary tests showed that *K. marmoratus* did not feed while out of water (M.W.W., unpublished observation). Embryo production was measured daily. At the end of the 96 h period, adults were returned to standard conditions for 1 week and then retested in the alternative environment.

Series II: impact of rearing environment on early development and metabolism

To determine the consequences of an aerial environment on embryonic development and metabolism, individual embryos (within 24 h of release from adults held under constant conditions) were reared in simulated aquatic and moist terrestrial environments (>99% relative humidity). It should be noted that *K. marmoratus* embryos are held within the adult for variable

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amounts of time, and approximately 20% of embryos are not released until 24–96 h post-internal fertilization (Harrington, 1963; Swain and Lindsey, 1986). To ensure that all embryos used for these experiments were newly fertilized and of approximately the same age, if any signs of organogenesis were evident (i.e. pigmentation), the embryos were not used. Air- or water-exposed embryos were maintained in the same manner as described above for adults. Filter paper was placed on the bottom of the water container to control for any confounding effects the paper may have on development. After 7, 15 and 30 dpr, embryos were manually dechorionated and then photographed under a dissecting microscope with a Venus 2.0 microscope camera (Am Scope, Irvine, CA, USA). Embryos were then blotted dry and wet mass was recorded with the chorion, and with the chorion and yolk sac removed (embryo only); dry mass (embryo only) was determined after drying to constant mass (40°C for 30 min). The percentage body water of embryos was calculated by dividing the water mass of the embryo (dry mass subtracted from wet mass) by the wet mass of the embryo.

All images were randomized to enable them to be analysed blind by the single observer (M.W.W.). Developmental stage was determined according to Mourabit et al. (2011). To determine the morphometrics of 30 dpr embryos, the standard length, body depth and yolk sac surface area were measured. Eight morphological landmarks were selected for measurements: (1) tip of the snout, (2) ventral point where the head meets the yolk sac, (3) most dorsal and posterior portion of the head, (4) posterior and proximal point of the operculum, (5) most dorsal point of the yolk sac, (6) most ventral point of the yolk sac, (7) where the posterior point of the yolk sac meets the tail and (8) posterior end of the tail before the caudal fin. Landmarks were digitized using TPS Dig2 (F. J. Rohlf, Department of Ecology and Evolution, State University of New York at Stony Brook, NY, USA). The total length of the embryo was determined by producing vectors using points 1–4–8. Body depth was determined using points 3–5. The yolk sac surface area was determined by creating two representative triangles on the images using points 2–5–7 and 2–6–7 and calculating the cumulative area.

To measure chorion morphology, embryos were reared in simulated aquatic or terrestrial environments for 30 days. Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C, and then moved to 30% sucrose in PBS for another 24 h at 4°C. Embryos were embedded in Shandon Cryomatrix resin (Fisher Scientific, Mississauga, ON, Canada) and cryosectioned in 10 µm thick sections. Sections were either stained routinely with hematoxylin and eosin to observe basic chorion morphology, or stained with the collagen-specific dye Picrosirius Red to observe just the collagen component of the chorion using the method of Johnson et al. (2014).

To measure the metabolic rate of embryos, a fibre-optic optode (PreSens Precision Sensing GmbH, Regensburg, Germany) and closed respirometry were used in both air and water. Oxygen consumption was measured in an air-tight respirometry chamber (volume 0.189 ml) constructed out of a small 12×32 mm glass screw neck vial (Waters, Milford, MA, USA). The vial was placed inside a glass chamber with circulating water (25±0.1°C). A small stir bar (~4 mm) was placed in the bottom to mix water or air throughout the experiment. A mesh stand held the embryo above the stir bar in water. For aerial oxygen consumption, the chamber volume was reduced by ~3/4 using paraffin wax. An air-tight lid screwed onto the chamber and a small hole allowed for insertion of the optode. To confirm that there was no leakage of oxygen into or out of the chamber, oxygen concentration of chambers filled with pure nitrogen or oxygen was measured over 3 h in preliminary experiments. The optode was calibrated to 100% DO saturation using brackish water (25°C, 16‰, pH 8) aerated with atmospheric air and 0% DO saturation using 2 mol l^{-1} Na_2SO_3.

For oxygen consumption measurements in water, the chamber was filled with well-aerated water and one water-reared embryo was added. For oxygen consumption measurements in air, the chamber was filled with atmospheric air and five embryos reared in a terrestrial environment were added. Metabolic rate in embryos was measured at 7, 15 and 30 dpr. Embryos were allowed to acclimate for 1 h in the vial before oxygen consumption was measured. Aerated water was slowly flushed through the chamber to ensure aquatic embryos remained in well-oxygenated water. For terrestrial embryos, the chamber was not sealed to allow gas exchange during the acclimation period (1 h). Oxygen consumption was measured for 1 h, with DO saturation always above 70%. Embryonic oxygen consumption was calculated using the rate of decrease in oxygen inside the closed chamber, mass of the embryo, volume of water inside the chamber and the duration of exposure. Oxygen consumption is presented as µmol O_2 g^{-1} h^{-1}.

To determine whether returning to an aquatic environment acutely affected the metabolism of air-reared embryos, the aquatic metabolic rate of *K. marmoratus* embryos reared in air or water was measured. Embryos reared in air for 30 dpr were placed in the water-filled chamber for a 1 h acclimation period (i.e. hour 0–1) before O_2 uptake was measured over the second hour in water (i.e. hour 1–2). Therefore, in the text and figure legends this timeline is referred to as 'after 1 h'. The metabolic rate of embryos (30 dpr, N=9) was determined as above using closed respirometry with the following exceptions. A custom-built respirometry chamber was constructed out of a 1.7 ml plastic microcentrifuge tube (Fisher Scientific). A stir bar (4 mm) was placed in the bottom of the tube to mix the water and a mesh stand was placed above the stir bar to avoid agitation of the embryos. A hole (~1 mm diameter) was punctured in the tip of the tube to allow insertion of the micro-oxygen electrode needle (OX-7245, Unisense, Aarhus, Denmark). The tube was sealed once the electrode was inserted using Blu-Tack. Preliminary tests confirmed that no oxygen leaked into the chamber. A picoammeter (PA2000, Unisense) was used to measure DO saturation via the relative amplitude of the water. These measurements were recorded using the program LabChart 6 through PowerLab 4/30 (AD Instruments, Colorado Springs, CO, USA). The micro-oxygen electrode was calibrated as described above before and after the experiment to account for any drift. A blank run was carried out to determine the relative rate of oxygen consumption of the electrode. Embryos were pooled in groups of five and two for water- and air-reared animals, respectively. It should be noted that the optode equipment was not available at the time of these experiments. However, in preliminary trials we established that oxygen uptake in water-reared embryos was very similar using the two different oxygen-detecting systems.

To measure lactate accumulation, whole *K. marmoratus* embryos reared in air or water for 30 dpr were first dechorionated, deyolked and then deproteinized in a 1:8 (mass:volume) solution of chilled 8% perchloric acid. The embryos were sonicated for 2–3 s on ice (model VC50T, Sonics and Materials Inc., Danbury, CT, USA), centrifuged at 15,000 ×***g*** for 10 min (4°C) and supernatant lactate concentrations were measured enzymatically (Bergmeyer, 1983).

Series III: impact of rearing environment on embryo movement

To test whether rearing environment impacts embryo movement, body and opercular movements in air and water were quantified at 7, 15 and 30 dpr. Individual embryos were placed in a Petri dish on a piece of wet filter paper for air-reared embryos or filled with 10±0.5 ml of brackish water (16‰, 25°C, pH 8) for water-reared embryos. Embryos were video recorded through a dissecting scope for 1 h. At 30 dpr, air- and water-reared embryos were placed in a Petri dish filled with brackish water. Movements of encapsulated embryos were categorized as either large body movements (Ciuhandu et al., 2007; Tattersall and Spiegelaar, 2008; Movie 1) or opercular movements. Values were calculated as the number of movements per hour. An additional group was added to determine whether returning to an aquatic environment affected the movement rate of air-reared embryos. This group of air-reared embryos were placed in water at 30 dpr and movement was measured in the same manner described above.

Series IV: environmental hatching triggers

Kryptolebias marmoratus embryos (N=8) reared in water for 30 dpr were used to determine whether acute changes in environmental conditions trigger hatching. Five conditions were tested: (1) normoxic brackish water (DO saturation >90%, 16‰), (2) normoxic freshwater (DO saturation >90%, 0.1‰), (3) hypoxic brackish water (DO saturation=10%, 16‰), (4) normoxic air (DO saturation >90%) and (5) hypoxic air (DO saturation=10%). The normoxic freshwater group was added as there are reports of *K. marmoratus* embryos hatching in response to rainfall (Taylor, 1990). Aquatic exposures were achieved by placing a single embryo in a 100 ml beaker filled with 80 ml of water and sealed with Parafilm (Bemis Company, Inc., USA). For the terrestrial exposure, embryos were placed in a

similar beaker on a piece of water-saturated filter paper. Oxygen content was established by aerating the beaker with either humidified atmospheric air for normoxia or nitrogen gas for hypoxia, and was monitored continuously with a dissolved oxygen probe (Vernier Software & Technology, USA). Embryos were video recorded for 60 min to determine time to hatching with minimal disturbance. Embryos that did not hatch within 60 min were considered to be non-responsive to the treatment.

Series V: long-term effects of rearing environment

Embryos exposed to either a terrestrial or aquatic environment (as described in series II) were raised to maturity (10 months). Pairs of isogenic embryos (one exposed to water, the other to air) were selected from a single clutch, allowing pairwise comparison. All embryos were dechorionated at 30 dpr and returned to standard aquatic holding conditions. The first day an embryo was released by the adult was recorded as the age at reproduction. Once adults were reproductively active, the number of embryos released by each adult was recorded for 5 months. After 10 months, adult mass (g) and standard length (cm) were measured. Adults were then air-exposed for 7 weeks in the same manner described above. The survival of adults was recorded daily. At the end of the 7 weeks of air exposure, any surviving fish were euthanised.

Statistical analysis

Statistical analyses were carried out using R 2.15.1 (R Core Team, 2012) with the critical α=0.05. Paired Student's *t*-tests were used to compare the reproductive output of adult mangrove rivulus in water and air at 24, 72 and 96 h. Two-way ANOVA with Holm–Sidak *post hoc* tests were used to determine whether air exposure had an effect on the mass of the embryo (intact, embryo wet, embryo dry and % body water). The morphological measurements at 30 dpr were analysed using two-sided Student's *t*-test. A two-way ANOVA with Holm–Sidak *post hoc* test was used to determine the effects of development in air or water on metabolic rate at 7, 15 and 30 dpr. Two-sided Student's *t*-tests for independence were run to determine the effects of terrestrial rearing on embryonic oxygen consumption when embryos were returned to water. The embryonic movement within the chorion was analysed using two-way ANOVA with Holm–Sidak *post hoc* tests. Preliminary analysis of the opercular movement residuals showed several high leverage points (Cook's distance >0.5) and, as such, a log transformation was applied to the number of opercular movements per hour. The mean oxygen consumption at each time point (7, 15 and 30 dpr) of embryos reared in air or water was regressed against the number of opercular movements per minute. The time to hatch for embryos that were exposed to the different environmental hatching triggers and successfully hatched (hypoxic water and hypoxic air) was compared using a two-sided Student's *t*-tests for independence. Paired *t*-tests were performed to determine whether rearing environment had an effect on adult age at reproduction, reproductive output (measured as the average number of embryos released per week over a 12 week period beginning with first reproduction), body mass, standard length or survival during emersion.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.W.W., A.J.T. and P.A.W. conceived and designed the project. M.W.W. executed most of the experiments, analysed the data, and wrote the draft manuscript. A.J.T. was responsible for experiments on adult fish and performed chorion histology. P.A.W., A.J.T. and M.W.W. revised the manuscript.

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Supplementary information

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