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No Water, No Problem: Stage-Specific Metabolic Responses to Dehydration Stress in Annual Killifish Embryos

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No water, no problem: Stage-specific metabolic responses to dehydration stress in annual killifish embryos

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Summary Statement

Annual killifish embryos survive over a year without water. Dormant and actively developing embryos exhibit opposite metabolic responses to dehydration. These responses may contribute to phenotypic variation associated with developmental bet-hedging.

Abstract

Annual killifish survive in temporary ponds by producing drought-tolerant embryos that can enter metabolic dormancy (diapause). Survival of dehydration stress is achieved through severe reduction of evaporative water loss. We assessed dehydration stress tolerance in diapausing and developing *Austrofundulus limnaeus* embryos. We measured oxygen consumption rates under aquatic and aerial conditions to test the hypothesis that there is a trade-off between water retention and oxygen permeability. Diapausing embryos survive dehydrating conditions for over 1.5 years, and post-diapause stages can survive over 100 days. Diapausing embryos respond to dehydration stress by increasing oxygen consumption rates while post-diapause embryos exhibit the same or reduced rates compared to aquatic embryos. Thus, water retention does not always limit oxygen diffusion. Aerial incubation coupled with hypoxia causes some embryos to arrest development. The observed stage-specific responses are consistent with an intrinsic bet-hedging strategy in embryos that would increase developmental variation in a potentially adaptive manner.

Abbreviations used:

DII = diapause II,

DO = dissolved oxygen

dpd = days post-diapause II

dpf = days post-fertilization

LT_{50} = lethal time to 50% mortality

P_{crit} = critical oxygen level

RH = relative humidity

MO_2 = oxygen consumption

WS = Wourms' stage

Introduction

Water is essential for life and the threat of dehydration due to evaporative water loss is ever-present in terrestrial organisms and especially in aquatic organisms that sometimes exploit terrestrial environments (Martin and Cooper, 1972; Martin and Carter, 2013; Martin et al., 2004; Martin, 1999; Moravek and Martin, 2011). Few aquatic organisms can exploit terrestrial habitats for prolonged periods of time, and many fish embryos that experience aerial conditions are found in moist environments for relatively short durations (days to weeks), therefore evading severe loss of water (Martin, 1999; Martin et al., 2011; McDowall and Charteris, 2006; Podrabsky et al., 2010b; Taylor, 1999; Thompson et al., 2017; Tingaud-Sequeira et al., 2009; Wells et al., 2015). Annual killifish embryos are an exception and can survive for months to years in dry mud (Polačik and Podrabsky, 2015). Survival of prolonged dehydration stress is invested solely in drought-tolerant embryos. Despite the clear importance of dehydration tolerance to the survival of annual killifishes, it remains unclear how these embryos can survive prolonged dehydration stress.

Survival through the dry season in annual killifishes is attributed to the ability of embryos to enter a state of metabolic dormancy (diapause) as a part of their normal development (Podrabsky and Hand, 1999; Podrabsky and Hand, 2000) and to resist evaporative water loss (Podrabsky et al., 2001). Embryos can enter diapause at three distinct stages termed diapause I, II, and III (Podrabsky et al., 2017; Wourms, 1972a; Wourms, 1972b). Diapause II (DII) is the most stress-tolerant stage and is likely responsible for long-term survival of dehydrating conditions (Podrabsky et al., 2016; Podrabsky et al., 2001; Polačik et al., 2020). When exposed to aerial conditions, embryos initially lose about 50% of their extraembryonic water in the first few days, but then water loss approaches zero and the embryo remains fully hydrated (Podrabsky et al., 2001). Dramatically reduced permeability to water vapor suggests highly limited gas exchange in general (Danks, 2000), and thus we predict that this mechanism for survival of dehydration stress may result in lower respiratory gas exchange and produce a self-imposed hypoxia or anoxia. Lack of oxygen (anoxia) is known to induce quiescence (halting development) in developing embryos of *A. limnaeus* (Podrabsky et

al., 2007), and thus we also investigated whether dehydration stress may directly or indirectly induce developmental arrest.

The annual killifish, *Austrofundulus limnaeus*, is an emerging model for developmental physiology and ecology because of their tolerance of environmental stresses, annotated genome, and the ability to manipulate developmental trajectories in the lab (Podrabsky et al., 2017; Podrabsky et al., 2010a; Romney et al., 2018; Wagner et al., 2018). Here we report on the survival and metabolic responses of diapausing and developing post-diapause II embryos under aerial conditions to evaluate their survival of dehydration stress. We discuss these data in relation to the embryo ecology of annual killifishes and suggest that long-term survival in the soil may be supported by entrance into diapause II, while intrinsic variation in responses to dehydration and oxygen stress may lead to a variety of developmental outcomes.

Materials and Methods

Animal husbandry and embryo collection

Adult annual killifish were housed and embryos collected as previously described (Podrabsky, 1999). This work was performed under established protocols which were approved and reviewed by the Portland State University Institutional Animal Care and Use Committee (PSU IACUC protocols #33 and 64). Briefly, adult annual killifish were kept in male-female pairs and spawned semiweekly. Embryos were collected and stored at 25°C with no light in 15 x 100 mm plastic Petri dishes in media that resembles the environmental conditions from which adults were collected in 1995 (10 mmol l^{-1} NaCl, 2.15 mmol l^{-1} $MgCl_2$, 0.8 mmol l^{-1} $CaCl_2$, 0.14 mmol l^{-1} KCl, 1.3 mmol l^{-1} $MgSO_4$) (Podrabsky, 1999; Podrabsky et al., 1998). During the first 4 days post-fertilization (dpf), embryo medium contained methylene blue (0.0001%) to prevent fungal infection. Embryos were then treated with two 5 min washes of a solution of 0.01% sodium hypochlorite (separated by a 5 min rest in embryo medium) to prevent bacterial and fungal growth, as previously described (Podrabsky, 1999). Following sodium hypochlorite treatment, embryos were transferred to embryo medium containing 10 mg

l^{-1} gentamicin sulfate and allowed to develop to DII (32–46 d). To break DII, embryos were subjected to a temperature of 30°C and full spectrum light for 48 h (Meller et al., 2012). Following this treatment, embryos were sorted into synchronized cohorts of embryos by developmental stage (Podrabsky et al., 2017).

Embryonic stages investigated

Experiments were performed on four developmental stages of embryos (Podrabsky et al., 2017; Wourms, 1972a; Wourms, 1972b) to capture a gradient of stress tolerance levels and physiology: Wourms' stage (WS) 32/33 or diapause II (DII), WS 36 (4 days post-diapause II (dpd)), WS 40 (12 dpd), and WS 42 (20 dpd). DII embryos are metabolically dormant and have halted development, whereas the other stages are metabolically active and developing. In all cases embryos were taken from a common pool of embryos produced by 42 spawning pairs of fish.

Tolerance of aerial exposure

Embryos (DII, WS 36, WS 40, WS 42) were exposed to 85% relative humidity (RH) air at 25°C with no light in a sealed plastic desiccator (Nalgene, 250 mm diameter). Relative humidity was controlled by using a saturated solution of potassium chloride (750 ml) placed below the shelf and continually mixed with a stir bar to ensure uniform RH within the aerial portion of the chamber (Podrabsky et al., 2001; Winston and Bates, 1960). Prior to dehydration exposure, embryos were treated with sodium hypochlorite (see above) and were allowed to recover for 2 h at 25°C without light. Groups of 20 embryos (N=3) per stage were removed from their aquatic medium and placed in Petri dishes (50 mm, PDF2047S0, Fisher Scientific, Hampton, NH, USA) containing a sterilized filter pad saturated with 2.5 ml of embryo medium containing 10 mg l^{-1} gentamicin sulfate (Podrabsky et al., 2001). Embryos were monitored until 100% mortality was reached. Embryos were determined dead when they lost their shape and shine. Survival between stages was compared by calculating lethal time to 50% mortality (LT_{50}) in response to aerial exposure.

Metabolic rate (respirometry)

Aerial incubation

Diapause II. Embryos were treated with sodium hypochlorite (see above) and incubated in embryo medium containing 10 mg l^{-1} gentamicin sulfate and 2.5 µg l^{-1} amphotericin B (10128-872, VWR, Radnor, PA, USA) for 24 h prior to aerial exposure. For aerial incubation, DII embryos were placed on filter pads containing 2.5 ml embryo medium containing gentamicin and amphotericin B using sterile technique within a biosafety cabinet. Care was taken to make certain that single embryos were isolated and not touching other embryos. DII embryos were then exposed to 85% RH at 25°C without light for 11 d to allow equilibration of the filter pad with the aerial environment. Four biological replicates (*N*=4), comprised of five embryos each, were placed in standard opening 2 ml borosilicate glass crimp-top vials (03-391-2, 12 OD x 32 mm long, Fisher Scientific, Hampton, NH, USA) containing a 4 mm RedEye oxygen sensor patch for non-invasive oxygen monitoring (Ocean Insight, Largo, FL, USA). Embryos were lifted off the filter pad using ring forceps and gently transferred into a vial that was pre-equilibrated with 85% RH air. No filter pad material was transferred with the embryos. Transfer of embryos was done within a biosafety cabinet using sterile technique. Vials containing embryos and sensor patches were crimped with aluminum crimp seals and a black Viton™ elastomer septa (03-378-343, Thermo Scientific, Waltham, MA, USA). Vials and septa were equilibrated at 85% RH for 30 min prior to use. To ensure sterilization of vials and sensor patches, vials containing patches were prepared in a biosafety cabinet and UV-sterilized for several hours prior to addition of embryos.

Wourms' stage 36 embryos. Diapause II embryos were treated with sodium hypochlorite and incubated in embryo medium containing 10 mg l^{-1} gentamicin sulfate and 2.5 µg l^{-1} amphotericin B for 24 h. Embryos were placed on filter pads containing 2.5 ml embryo medium with gentamicin and amphotericin B using sterile technique and placed into a desiccator at 85% RH (see details above). Diapause II was broken under aerial incubation by placing the desiccator under full spectrum light in an incubator at 30°C. After 72 h, temperature was reduced to 25°C and the light was turned off. Following 24 h of 25°C without light, embryos were removed and staged. Filter pads were then

weighed to ensure equilibration with chamber relative humidity and showed no difference with the filter pads used for DII treatments. For WS 36 embryos, 7 biological replicates (*N*=7), comprised of five embryos each were used. Embryos were added to 2 ml vials containing sensor patches and crimp sealed under sterile conditions as described above.

Aquatic incubation

Diapause II and WS 36 embryos were sorted and treated with sodium hypochlorite as described above. Following treatment, embryos were allowed to recover for 2 h prior to the start of the respirometry experiment. Embryo medium containing 10 mg l^{-1} gentamicin sulfate was aerated by gentle bubbling with atmospheric air at 25°C for 1 h to ensure dissolved oxygen (DO) saturation. To prevent supersaturation, medium was allowed to equilibrate for 5 min without aeration prior to use. Embryo medium was then added to vials containing 5 embryos each (DII, *N*=4; WS 36, *N*=5) and a sensor patch. Vials were immediately crimp sealed with Viton™ caps making sure to leave no air bubbles trapped within the vials.

Oxygen measurement and embryo observation

Measurements of sensor patch fluorescence were made through the vial wall using a fiber optic probe in conjunction with a NeoFox Oxygen Sensing System (Ocean Insight, Largo, FL, USA). For aquatic conditions, the system was calibrated to 100% DO saturation using vials containing embryo medium aerated with atmospheric air at 25°C and 0% DO saturation using embryo medium bubbled with N_2 for 30 min and equilibrated overnight in a Bactron III anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA), containing 5% CO_2, 5% H_2, and 90% N_2. For aerial conditions, the system was calibrated to 100% saturation using vials containing atmospheric air at 25°C and 0% saturation using 5% CO_2, 5% H_2, and 90% N_2 from the anaerobic chamber. Previous observations illustrated no effects of the anerobic gas mixture on fluorescence measurements (data not shown). Vials sealed at 0% and 100% saturation (both aerial and aquatic) were regularly measured alongside samples. These control vials remained consistent across the entire duration of the experiments, and were used as blank vials

to control for the possibility of contamination and to ensure stable calibration of the NeoFox Oxygen Sensing System. Oxygen concentration was monitored regularly and oxygen consumption rate was used as a proxy for metabolic rate. All measurements were taken at 25°C with minimal light. Following measurement of oxygen concentration, embryos were observed under a dissection microscope (Leica, S8 APO, Wetzlar, Germany) to record health and developmental stage. Rate of oxygen consumption was calculated using the slope of decrease in oxygen content inside each vial over time divided by the number of embryos in the vial. Oxygen consumption is presented as nmol O_2 h^{-1} $embryo^{-1}$.

Statistical analysis

Graphical and statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA) and SPSS software (IBM., v.26.0, Armonk, NY, USA). LT_{50} of embryos exposed to dehydration was calculated for each developmental stage by probit regression analysis (Chapman et al., 1995) using SPSS software. Where appropriate, analysis of variance (ANOVA), unpaired t-test, or linear regression analyses were used. Tukey's honest significant different (HSD) test was used for *post-hoc* comparisons, where applicable. Statistical significance was set to $P<0.05$ for all comparisons.

Results and Discussion

Despite knowledge of the unprecedented ability of annual killifish embryos to survive long periods of aerial exposure, this is the first report of lethal time to 50% mortality (LT_{50}) values measured under controlled conditions. Tolerance of aerial conditions was greatest in DII embryos, with 10% surviving over 500 d and a single embryo living for 587 d (Fig 1A), and decreased during post-DII development. Remarkably, all post-DII stages had embryos that survived longer than 100 d, although the LT_{50} values were around a month of exposure for late-stage embryos. The ability of these embryos to survive in 85% RH for hundreds of days is impressive for an aquatic

embryo especially considering that 85% RH is equivalent to being bathed in a solution of around 8000 mOsmol kg^{-1} salt (around 8X seawater). This ability is almost unparalleled in any other aquatic species and certainly suggests unique adaptations for preventing evaporative water loss that rivals terrestrial eggs.

The long-term survival of DII embryos during aerial incubation may require physiological responses to both prevent and respond to evaporative water loss. The egg envelope, perivitelline space, and enveloping cell layer of embryos likely work in concert to resist water loss and support survival (Podrabsky et al., 2001), and molecular changes to these structures are of interest in explaining the extreme resistance to water loss. Over time the embryonic compartment eventually loses enough water that the embryos die (Podrabsky et al., 2001), and thus mechanisms, as observed in other species, may be induced to help stabilize cellular components and preserve macromolecules as water content slowly declines (Bayley and Holmstrup, 1999; Bayley et al., 2001; Lopez-Martinez et al., 2009). It is worth noting that accumulation of hygroscopic compounds is a very unlikely mechanism for survival of *A. limnaeus* embryos as this response is only effective at very high RH (>98%) and previous reports of exposure to aerial dehydration stress indicate bulk water in the embryonic compartment and not structured water as would be expected if high amounts of osmolytes were accumulating (Podrabsky et al., 2001).

It is likely that *A. limnaeus* populations survive the dry seasons through entrance into DII (Podrabsky et al., 2016; Podrabsky et al., 2001). A field survey of African annual killifish embryos (genus *Nothobranchius*) revealed an egg bank primarily of DII embryos during the peak dry season (Polačik et al., 2020). This is consistent with the remarkable tolerance of DII embryos to aerial conditions. However, even the lower tolerance of WS 36 embryos is impressive and suggests induction of mechanisms that could support survival through a typical dry season if soil conditions remain at 85% RH or greater.

Diapause II embryos consumed the available oxygen in aquatic vials faster, but at a slower rate than in aerial vials (Fig 2A,C,E; 33 times lower available oxygen in water compared to air). Aerial diapause II embryos had an oxygen consumption rate that was over 4 times higher than aquatic embryos despite remaining in diapause for the duration of the experiment (Fig 3A). These results were even more surprising given

that under aerobic aquatic conditions, DII embryos support a large portion of their metabolism via anaerobic pathways (Podrabsky and Hand, 1999; Podrabsky et al., 2012). These data suggest either an energetic cost of survival of aerial conditions, or a diffusion-limited metabolism under aquatic conditions. Like all fish embryos prior to hatching, *A. limnaeus* embryos are limited to diffusive gas exchange with their environment. Under conditions of stagnant water flow, this can lead to large boundary layers (up to 5 mm) that may limit diffusion rates (DiMichele and Powers, 1984). Further, the thick egg envelope of annual killifishes (Schoots et al., 1982) may also contribute to limited rates of diffusion. When exposed to aerial conditions, embryos of *A. limnaeus* lose most of their perivitelline water within the first week (Podrabsky et al., 2001), which effectively brings the egg envelope closer to the fully hydrated embryonic compartment and may reduce the diffusion distance for gas exchange. The diffusion coefficients through the egg envelope under hydrated and aerial conditions are unknown and would need to be determined to evaluate if diffusion may be limiting gas exchange. However, this explanation is unlikely given the low metabolic demand for oxygen in DII embryos. Further, developing embryos of *Fundulus heteroclitus* (DiMichele and Powers, 1984) do not exhibit diffusion-limited metabolic rates in aquatic conditions until extremely late in development and do not experience an increase in oxygen consumption under aerial conditions. Thus, it is much more likely that the increased oxygen consumption rate in DII embryos represents the cost of a physiological response that requires reactivation of suppressed metabolic pathways needed for survival of dehydration stress.

All post-DII embryos began respirometry experiments at WS 36; however, the duration of aquatic exposures was short due to exhaustion of oxygen in the vials (Fig 2B). Initially, post-DII embryos (WS 36) of *A. limnaeus* maintained a similar oxygen consumption rate in aerial and aquatic conditions (Fig 3A). This result is consistent with previous reports for *F. heteroclitus* (DiMichele and Powers, 1984; Tingaud-Sequeira et al., 2009). Initially, post-diapause II embryos developed synchronously, but then developed at different rates after 7 days of aerial exposure (Fig 3C) leading to a divergence in oxygen consumption rates between replicates over time (Figs 2D,E, 3C). Late-stage aerial incubated embryos had a significantly higher oxygen consumption rate

than initial rates observed in aerial and aquatic treatments, as would be expected for continually-developing embryos (Fig 3B). However, mean oxygen consumption rates in aerial WS 42 embryos that developed synchronously and continuously were significantly lower (60% lower) compared to those reported for aquatic conditions in previous studies (Fig 3A). This result for late stage embryos is consistent with observations in *Kryptolebias marmoratus* which experience a 44% reduction in oxygen consumption rate in response to aerial exposure (Wells et al., 2015). It is important to note that oxygen consumption rates in *A. limnaeus* begin to diverge (Fig 2D) at PO_2 values well above the measured P_{crit} of *A. limnaeus* embryos under aquatic conditions (Podrabsky and Wilson, 2016). Thus, lower rates of oxygen consumption are not likely due to an acute response to hypoxia, but more likely represent a physiological response (metabolic depression) to a mixture of dehydration stress and hypoxia.

While the majority of the aerial embryos continued developing similar to their aquatic counterparts, a subset (6/35, 17%) of the embryos in four of the experimental vials remained at WS 38 from day 7 until the completion of the experiment at day 19. Embryos of *A. limnaeus* enter quiescence in response to anoxia (Podrabsky et al., 2007), but this is the first report of quiescence-like behavior in response to aerial conditions. It is likely that this stalling of development is due to a combination of mild hypoxia and aerial exposure, because embryos exposed to 85% RH in large containers where oxygen is not limited tend to develop synchronously (D.E.Z., personal observation). Of great interest is the apparent intrinsic variation observed in response to the aerial incubation conditions, which leads to developmental arrest in only a proportion of embryos. Asynchronous development could have profound influences on the developmental ecology of this species. A pool of embryos in the soil at different developmental stages could be advantageous for species persistence by reducing the probability of total recruitment failure driven by false environmental cues of an imminent rainy season (Polačik et al., 2020). Thus, this could represent a form of intrinsic bet-hedging and a source for added variation in the egg bank that increases the probability of population survival in a given location (Furness et al., 2015). Since stress tolerance decreases during post-DII development (Machado and Podrabsky, 2007; Podrabsky et

al., 2007; Riggs and Podrabsky, 2017; Wagner et al., 2019), slowing developmental rate during aerial exposure may prolong the period of stress tolerance and increase survival.

In summary, we show for the first time that embryos of *A. limnaeus* can survive aerial incubation for well over a year. If evaporative water loss is achieved through a general decrease in membrane permeability, aerial incubation should limit respiratory gas exchange and lead to self-imposed hypoxia or anoxia. While results for late-stage embryos are consistent with this hypothesis, DII embryos respond in an opposite manner. This leads to an especially interesting question of how these embryos increase their oxygen consumption rate while simultaneously reducing evaporative water loss to near zero for over a year in extremely dehydrating conditions. These unique responses to dehydration in dormant versus developing embryos may have important implications for the ecology of embryos in the wild.

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Competing interests

The authors declare no competing interests.

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Figures

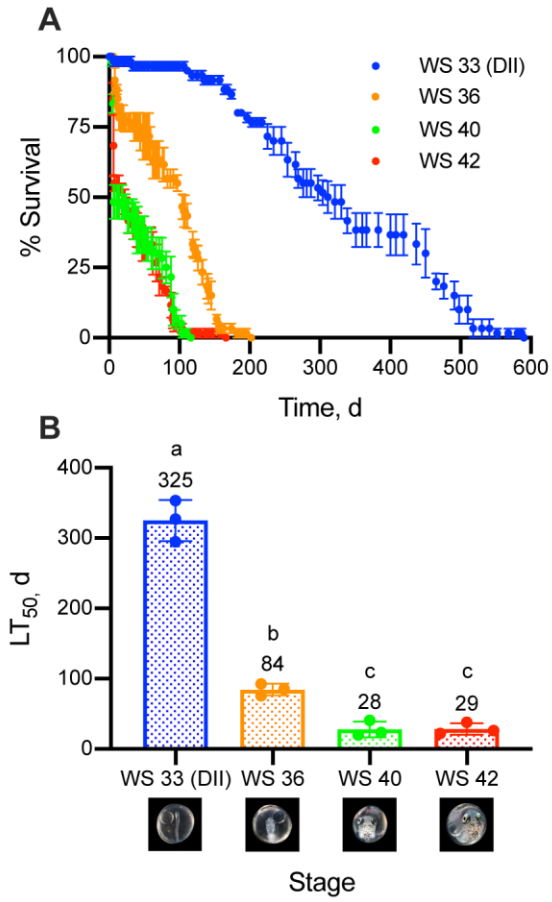
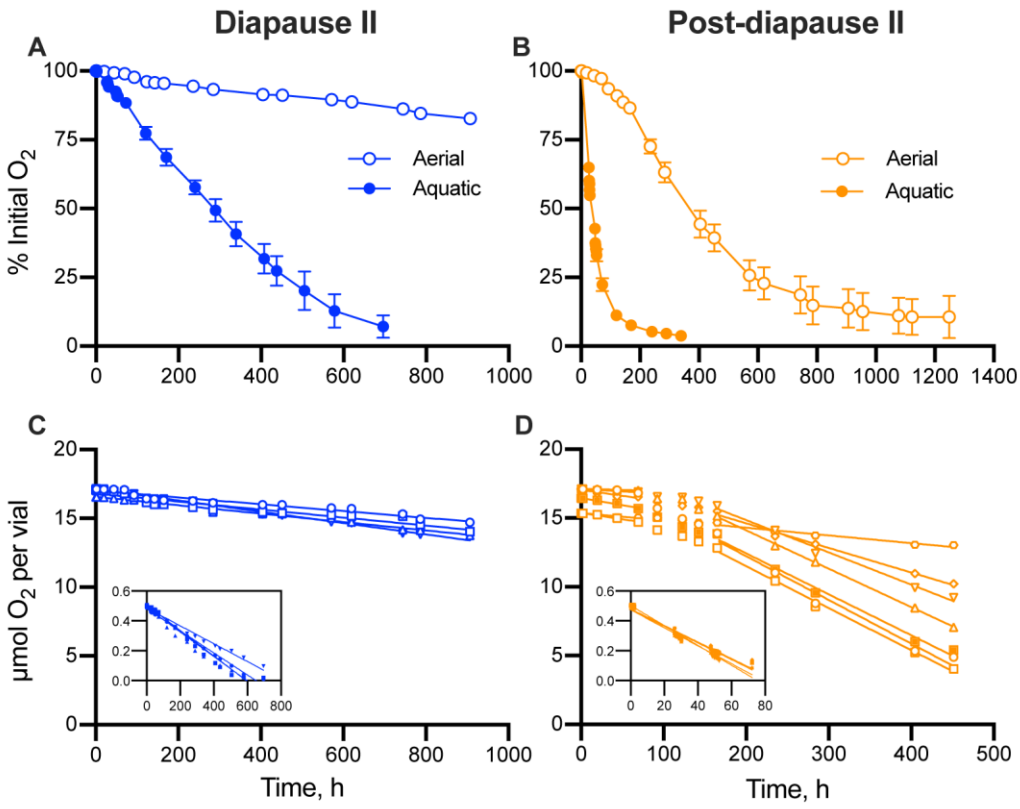


Figure 1. Survival of *Austrofundulus limnaeus* embryos incubated in aerial conditions. (**A**) Groups of 20 embryos were exposed to 85% RH at 25°C without light at the developmental stage listed. Diapause II embryos remained in diapause whereas post-DII embryos continued developing. WS, Wourms' stage; DII, diapause II. Symbols are means ± SEM (*N*=3). (**B**) Lethal time to 50% mortality (LT_{50}) for embryos exposed to aerial conditions. Different letters indicate significant differences in LT_{50} between stages (one-way ANOVA with Tukey's HSD test, $P<0.05$). Bars are means ± SEM (*N*=3) and symbols represent individual replicates.



E

MO_2 (nmol O_2 h^{-1} $embryo^{-1}$) of embryos incubated in aerial or aquatic conditions

		Diapause II		Post-diapause II			
Replicate	Parameter	Aerial	Aquatic	Aerial		Aquatic	
		Overall	Overall	WS 36	WS 38-42	WS 36	WS 38-42
1	MO_2	0.495	0.148	0.690	6.36	1.11	
	R^2	0.94	0.99	0.86	0.97	0.95	n /a
	P-value	< 0.0001	< 0.0001	0.0075	0.0017	< 0.0001	
2	MO_2	0.582	0.170	1.82	6.11	1.13	
	R^2	0.92	0.97	0.98	0.99	0.96	n /a
	P-value	< 0.0001	< 0.0001	0.0001	0.0001	< 0.0001	
3	MO_2	0.610	0.164	0.340	5.58	1.20	
	R^2	0.99	0.96	0.99	0.99	0.98	n /a
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
4	MO_2	0.823	0.120	0.660	4.69	1.33	
	R^2	0.99	0.98	0.88	0.99	0.97	n /a
	P-value	< 0.0001	< 0.0001	0.0005	0.0005	< 0.0001	
5	MO_2			1.71	3.58	1.11	
	R^2			0.92	0.99	0.94	n /a
	P-value			0.0025	0.0004	< 0.0001	
6	MO_2			1.046	1.12		
	R^2			0.94	0.95		
	P-value			0.0012	0.0048		
7	MO_2			2.05	5.95		
	R^2			0.98	0.99		
	P-value			0.0002	0.0006		

Figure 2. Oxygen consumption of *Austrofundulus limnaeus* embryos in aerial and aquatic conditions. (**A,B**) Percentage of initial oxygen in the closed system respirometry vials over time. Symbols are means ± SEM (*N*=4–7) and each replicate contained 5 embryos per vial. (**C,D**) The amount of oxygen (µmol) in each aerial exposure vial over time. Inset graphs are for aquatic conditions. Oxygen consumption rates were based on the slope of oxygen consumed over time. For DII embryos, a single

slope was used for the entire time-course for both conditions. For post-diapause II embryos, an initial WS 36 (aerial, 0–69 h; aquatic, 0–72 h) and late-stage slope (aerial, 165–452 h) were calculated. For aerial embryos, timepoints were omitted from regression analyses due to oxygen content not changing linearly (69–165 h) or because oxygen content was below the measured critical oxygen level (P_{crit}) of *A. limnaeus* embryos (after 452 h) (Podrabsky and Wilson, 2016). (**E**) Results from the regression analyses for each biological replicate used to calculate oxygen consumption rates.

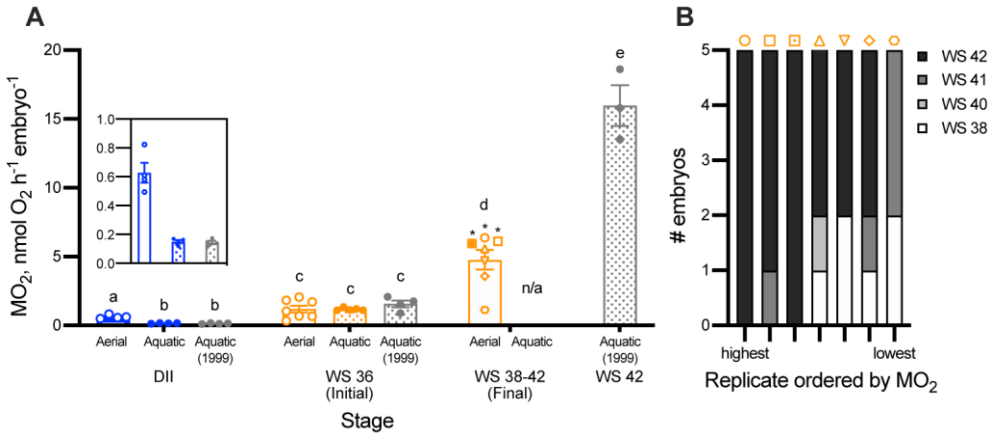


Figure 3. Rate of oxygen consumption in *Austrofundulus limnaeus* embryos exposed to aquatic and aerial conditions. (**A**) Diapause II embryos exposed to aerial conditions had a significantly higher oxygen consumption rate than those exposed to aquatic conditions (one-way ANOVA with Tukey's HSD, $P<0.0001$). Inset graph shows DII rates on an expanded scale. Wourms' stage 36 embryos (initial rate) exposed to aerial conditions had a mean oxygen consumption rate similar to those incubated in aquatic conditions (one-way ANOVA with Tukey's HSD, $P>0.05$). Oxygen consumption rates in late-stage embryos under aerial conditions varied considerably by replicate due to differences in developmental rates in embryos within each replicate as indicated by (**B**) the developmental stage of embryos in each vial at the end of the experiment. Replicates that developed synchronously to WS 42 in aerial conditions (replicates with asterisks in **A**) had significantly lower oxygen consumption rates than WS 42 developing in aquatic conditions reported in Podrabsky and Hand (1999) (unpaired t-test, $P=0.003$). Different letters indicate significant differences in rates of oxygen consumption (one-way ANOVA with Tukey's HSD test, $P<0.01$). For late-stage embryo measurements, symbols in **A** and **B** correspond to those used in Figure 2D for post-DII embryos. Bars in **(A)** are means ± SEM ($N=4$) and symbols represent individual replicates. Oxygen consumption rates for aquatic embryos from Podrabsky and Hand (1999) are included to demonstrate similar values despite use of different respirometry techniques.