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Phenotypic differences in terrestrial frog embryos: effect of water potential and phase

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SUMMARY

The terrestrial embryos of many amphibians obtain water in two ways; in a liquid phase from the substrate on which eggs are deposited, and in a vapour phase from the surrounding atmosphere. We tested whether the mode of water flux (liquid or vapour) affected the morphology and metabolic traits of the terrestrial Victorian smooth froglet (*Geocrinia victoriana*) embryos by incubating eggs both with a liquid water source and at a range of vapour water potentials. We found that embryos incubated with a liquid water source (ψ_π=0 kPa) were better hydrated than embryos incubated with a vapour water source (ψ_v=0 kPa), and grew to a larger size. Eggs incubated in atmospheres with lower ψ_v values showed significant declines in mass and in the thickness of the jelly capsule, while embryos primarily showed reductions in dry mass, total length, tail length and fin height. The most significant deviations from control (ψ_v=0 kPa) values were observed when the ψ_v of the incubation media was less than the osmotic water potential (ψ_π) of the embryonic interstitial fluid (approximately –425 kPa). Despite the caveat that a ψ_v of 0 kPa is probably difficult to achieve under our experimental conditions, the findings indicate the importance for eggs under natural conditions of contacting liquid water in the nesting substrate to allow swelling of the capsule.

Key words: water balance, water potential, osmotic pressure, vapour pressure, relative humidity, isopiestic, embryo, *Geocrinia victoriana,* Myobatrachidae, Anura, amphibian.

INTRODUCTION

Anuran amphibians reproduce in highly diverse ways, including depositing eggs in terrestrial nests (Altig and Johnston, 1989). In terrestrial breeding species without parental care, selection of nest sites with adequate water availability is critical, as eggs lose water freely from their jelly capsule and are at risk of desiccation (Bradford and Seymour, 1988; Mitchell, 2002). The jelly capsule consists of a matrix of acid mucopolysaccharides, which swells in the presence of water to form a liquid water reservoir (Salthe, 1963; Beattie, 1980) and buffers embryonic water loss in the short term (days). However, high mortality can result from prolonged incubation in terrestrial environments with less than optimal water availability (Martin and Cooper, 1972; Bradford and Seymour, 1988). Further, the growth rate of embryos that develop terrestrially is reduced in dry conditions and embryos have a lower tissue water content, metabolise less yolk and may have lower mass-specific rates of oxygen consumption (Taigen et al., 1984; Bradford and Seymour, 1988; Mitchell, 2002).

When eggs are first oviposited, the egg (or perivitelline) membrane tightly binds the ovum. After fertilisation, the perivitelline membrane separates from the ovum, creating a fluid-filled space that allows the embryo to rotate freely (Salthe and Duellman, 1973; Elinson, 1987). Under conditions of water stress, the volume of the perivitelline space does not increase and the jelly capsule swells only slightly. Embryos developing within smaller capsules have a reduced surface area for gas exchange and may risk becoming hypoxic, especially during the later stages of development when $\dot{V}_{O_2}$ is relatively high. Other damaging effects, including body asymmetry and head depression, may result from development in a dehydrated egg if the embryo is unable to rotate freely within the perivitelline space, and tail lesions can occur if the embryo adheres to the perivitelline membrane (Bradford and Seymour, 1988; Mitchell, 2002). In anuran species in which embryos hatch into a water body to become free-swimming, feeding tadpoles, such developmental anomalies will potentially be detrimental to swimming performance and may enhance predation risk.

The driving force for water movement is decided by the free energy (G) of a mass of water, such that water will move spontaneously from high water potential to low water potential (see Appendix). For aquatic frog eggs, osmotic water potential (ψ_π) is the driving force for exchange of water between the embryo and its environment, with the perivitelline membrane acting as a semi-permeable membrane. However, in terrestrial eggs, the matric (ψ_m), vapour (ψ_v) and osmotic components of water potential are all important drivers of water flux, and both liquid and vapour sources of water can be incorporated into the egg.

Embryos of the Victorian smooth froglet, *Geocrinia victoriana* (Anura: Myobatrachidae; Boulenger 1888) from southeastern Australia develop in moist depressions under leaf litter or grass on the periphery of water bodies that flood following winter rains (Littlejohn and Martin, 1964). Water availability for the embryos will be variable during development. At times the eggs may be submerged or with no liquid water available in the nest. Hatching is stimulated by full submersion of the egg during the winter rains (Martin and Cooper, 1972). Other species of *Geocrinia* (*G. leai; G. laevis*) deposit their eggs in elevated situations, usually attached to grass stalks (Main, 1965; Martin and Cooper, 1972); hence, this genus is an excellent model for examining the relative importance of the matric, vapour and osmotic components of water flux in terrestrial anuran eggs. The relative humidity (RH) above

G. victoriana egg masses developing in natural nests ranges between 85 and 100%, depending on nest exposure (Martin and Cooper, 1972). Depending on soil matric and osmotic properties (which were not measured), these humidities could potentially result in relatively low water potentials and consequently low water availability to the embryos. The survival limit of terrestrial anuran embryos incubated on filter paper substrates ranges between –25 kPa (*Bryobatrachus nimbus*) and –400 kPa (*Pseudophryne bibronii*) (Bradford and Seymour, 1988; Mitchell, 2002).

In this study we compared the morphology and metabolic traits of *G. victoriana* embryos incubated at a range of low vapour water potentials with those of embryos incubated in contact with liquid water.

MATERIALS AND METHODS

Study site and egg collection

Our study site was in remnant eucalypt bushland surrounding an ephemeral water body at Park Orchards, near Melbourne, Victoria (latitude: –37 deg. 47′ 0.78″S, longitude: 145 deg. 12′ 0.23″E, altitude 140 m). Six *G. victoriana* egg clutches were collected from the site during Autumn (April) and four clutches were obtained from matings between captive adults collected from the study site. Clutches were transported from the study site to the laboratory on damp tissue and refrigerated overnight at 4°C to limit development before they were used in an experiment. Two Thermochron iButton data loggers (DS1921, Maxim integrated products, Sunnyvale, CA, USA) recorded temperature (±0.05°C) every half hour in two representative nests at the study site during the period when eggs would be naturally incubated, from 22 April to 4 June, 2004.

Individual eggs were cleaned of debris by gentle rolling on damp tissue, and total clutch size, number of live embryos and developmental stage (Gosner, 1960) were recorded. Diameters of the yolk, perivitelline membrane and capsule were measured while embryos were briefly submerged (1–2 min) in distilled water (to prevent refractive errors), using an ocular micrometer under a stereomicroscope. This process did not appear to change the initial egg mass. Developmental stage of the eggs ranged between Gosner stages 1 and 9, and embryos were estimated to be no more than 2 days old when entering the experiment. Eighty-four eggs selected randomly from each egg mass were used in the experiments.

Establishment of water potential treatments

Previous studies of the effects of water potential on the embryonic development of frogs and reptiles have used wetted substrates (e.g. filter paper or vermiculite) for which a relationship between substrate water content and ψ_m had previously been determined (e.g. Miller and Packard, 1992). In contrast, ψ_v can be controlled by allowing vapour pressure (P_{H_2O}) to come into equilibrium with a reference solution of known molality; often referred to as the isopiestic technique (Solomon, 1951; Winston and Bates, 1960; Muth, 1977).

We established six ψ_v treatments between approximately –20 and –550 kPa (Table 1) using appropriate molalities of NaCl solutions calculated from van 't Hoff's equation for an incubation temperature of 12°C (see Appendix). Deionised water was used as the control treatment (ψ_v=0 kPa). Approximately 500 ml of each NaCl solution (or deionised water) was poured into a 1-l airtight container, and a 5 mm×5 mm stainless steel mesh shelf covered with two layers of Kimwipe® (WypAll® X50, Kimberly-Clark, NSW, Australia) was suspended approximately 20 mm above the liquid. Two eggs from each egg mass were placed in each container on the Kimwipe® into a labelled, 13 mm diameter nylon plumbing olive, and Vaseline® was smeared around the lids of all incubation chambers to ensure they were airtight. A second 0 kPa treatment in which eggs were able to contact liquid water (ψ_π=0 kPa) was also created. In this case the mesh shelf was set at the bottom of the container and was flooded with deionised water to a depth of ~1 mm. Six replicates of each of the eight treatments were randomly arranged on a shelving unit in a controlled-temperature (CT) room at 12±1°C (L:D, 11 h:13 h), close to the field-measured temperature (see Results), with one container from each treatment per shelf. Mean temperature ±0.2°C measured inside the containers ranged from 11.7°C on the bottom to 12.2°C on the top shelf. While this temperature variation for a given ψ_v may result in changes in RH of 1–2%, the experimental design ensured that there was a container from each treatment on each shelf and, thus, any changes in RH were consistent across all treatments.

Table 1. Vapour pressure (P_a) in the controlled atmospheres at 12°C above NaCl solutions achieved using the isopiestic technique and their associated vapour water potential (ψ_v) and relative humidity (RH)

NaCl (g kg^{-1} H_2O)	Osmolality (osmol kg^{-1})	P_a (kPa)	ψ_v (kPa)	RH
0	0	1.4013 (=P_S)	0	1.0000
0.0047	0.0093	1.4011	–22	0.9998
0.0109	0.0218	1.4008	–52	0.9996
0.0221	0.0442	1.4002	–105	0.9992
0.0434	0.0868	1.3991	–206	0.9984
0.1037	0.2074	1.3961	–493	0.9963
0.1121	0.2242	1.3957	–533	0.9960

Osmolality was measured with an osmometer (Vapro 5520). ψ_v and RH were calculated using equations from the Appendix.

The incubation chambers were prepared >3 weeks before any egg clutches were collected. The Kimwipe® was assumed to have reached the ψ_m of the NaCl solution (or H_2O) in less than 24 h; preliminary testing by following the mass changes in the Kimwipe® revealed that the Kimwipe® reached the maximum achievable water content for a given ψ_v in less than 1 day.

Egg clutches were collected over a 10 day period and thereafter containers were continuously sealed ensuring ψ_v was undisturbed. After 47 days of incubation the oldest embryos reached hatching stage 26 (Gollmann and Gollmann, 1991) and were removed for measurement of morphological traits and rate of oxygen consumption ($\dot{V}_{O_2}$). Removal of embryos from the remaining clutches occurred over the next 10 days when each clutch was 47 days old.

Morphological measurements

The diameters of the capsule and perivitelline membrane of embryos at Gosner stage 26 were measured using a stereomicroscope with an ocular micrometer while eggs were briefly submerged in distilled water. The eggs were then blotted on damp tissue, weighed on a Mettler AE240 balance (Columbus, OH, USA), and the embryos hatched by gentle rolling on damp tissue to remove the capsule layers. Embryos were immediately preserved in Tyler's fixative (Tyler, 1962). Dorsal and lateral photographs of the preserved embryos were taken using a digital camera attached to the stereomicroscope and measurements of total length, snout–vent length and tail lengths, heights and widths were made from the images using tpsDig 1.31 image analysis software (F. J. Rohlf, State University of New York, Stony Brook, NY, USA). Measurements of 10 live embryos were compared with those made of the same

embryos preserved for 4 weeks (approximately the time experimental tadpoles were fixed) to test for any effect of the fixative on tadpole dimensions. The gut was dissected from the body and carcasses were oven dried for 1 h at 50°C, then stored over silica gel for 3–4 days before dry masses were recorded (Mitchell and Seymour, 2000).

Rate of oxygen consumption

$\dot{V}_{O_2}$ was determined for unhatched stage 26 embryos from selected treatments (ψ_π=0 kPa, ψ_v=0, –22, –105 and –493 kPa) prior to morphological measurements by measuring the decay in the partial pressure of oxygen (P_{O_2}) in a sealed chamber with a Clark electrode (OXY040A, Rank Brothers Ltd, Cambridge, UK). The electrode was positioned at the bottom of the chamber and polarised with a current of 60 mV, and a rotating magnet caused a magnetic stirrer to rotate continuously, eliminating any boundary layer formed by electrode oxygen consumption. The embryos were placed in 5% Holtfreter's solution (approximately isotonic with pond water) on a stainless steel mesh stage above the stirrer, and the chamber was maintained at 12±0.2°C using water pumped from a water bath though a surrounding jacket.

Electrodes were calibrated at the beginning and end of each run with air-bubbled 5% Holtfreter's solution (P_{O_2} ~20.81 kPa) and electrical zero was used to indicate P_{O_2}=0 kPa. Any drift that occurred was assumed to be linear. P_{O_2} was recorded at 1 Hz (Chart, Powerlab AD Instrument, Bella Vista, NSW, Australia) and linear regression used to determine the change in P_{O_2} with time, after correcting for baseline drift. Embryonic $\dot{V}_{O_2}$ (in $\mu l\,h^{-1}$) was calculated from Eqn 1:

$$V_{O_2} = \frac{\text{Embryo}\Delta P_{O_2}}{\Delta t}(P_B - P_S)\times\beta_{O_2}\times\text{Vol}\times 0.2093\times 22.39\ , \quad (1)$$

where P_B is the barometric pressure (kPa), P_S is the saturation vapour pressure of water (1.40 kPa at 12°C), β_{O_2} is the capacitance of water for oxygen (22.23 $\mu mol\,l^{-1}\,kPa^{-1}$ at 12°C), Vol is the chamber volume minus the embryo volume (in liters, assuming 1 g wet mass=1 ml), 0.2093 is the fractional concentration of oxygen in well-aerated water, and 22.39 converts μmol to μl.

$\dot{V}_{O_2}$ was then calculated on a mass-specific basis using gut-free dry body masses (see above).

Osmolality of perivitelline and interstitial fluid

Samples of perivitelline fluid were collected from spare stage 26 embryos that were incubated at 12±0.5°C on tissue flooded with distilled water. The outer capsule layers were removed by gentle rolling on damp tissue, and the embryo was immersed in paraffin oil and perivitelline fluid collected in 10 μl glass microcapillary tubes after rupturing the perivitelline membrane with a needle.

Embryonic interstitial fluid was collected from 15 stage 26 embryos killed by immersion for 5 min in MS222 (150 $mg\,l^{-1}$). Immersion time was short to prevent any disturbance of osmotic balance. Groups of four to five embryos (to obtain sufficient volumes) were blotted on damp tissue, their tails were removed, and heads and tails were separately homogenised in 1.5 ml Eppendorf tubes using a Teflon pestle. The Eppendorfs were centrifuged for 3 min and the supernatant collected in microcapillary tubes. The osmolality of 10 μl samples of the perivitelline fluid and interstitial fluids was measured with a vapour pressure osmometer (Vapro 5520, Wescor, Logan, UT, USA), and ψ_π calculated using Eqn A1 (see Appendix).

Analysis

Data are presented as means ± 1 s.e.m., and percentage data were arcsine transformed for statistical analysis. Weighted linear regressions and statistical comparisons were performed using Minitab (v14), but the liquid water treatment (ψ_π=0 kPa) was not included in the regressions as water was exchanged with the embryo in a liquid phase rather than a vapour phase. For other analyses the saturated water vapour treatment (ψ_v=0 kPa) was treated as the control and all comparisons were made to this treatment (ANOVA with Dunnett's comparisons). Statistical significance was assumed at P<0.05.

RESULTS

Natural nest temperatures and egg clutch parameters

The temperatures of two *G. victoriana* nests ranged between 5.0 and 17.8°C with a mean of 10.9±0.03°C. Clutches ranged in size from 56 to 232 eggs with a mean of 113±11 eggs (N=18). The proportion of viable embryos in each clutch averaged 89.7±5.3%

Table 2. Linear regressions describing the relationship between water potential and a range of parameters measured in embryos from the vapour pressure treatments

Variable	a	b	S_a	S_b	S_{yx}	R^2	P
Egg mass (g)	2.17×10^{-2}	1.38×10^{-5}	5.48×10^{-4}	1.58×10^{-6}	1.04	0.94	<0.001*
Perivitelline membrane diameter (mm)	3.16	7.19×10^{-4}	2.19×10^{-2}	9.35×10^{-5}	7.42×10^{-1}	0.92	0.001*
Capsule diameter (mm)	3.64	1.08×10^{-3}	4.11×10^{-2}	1.55×10^{-4}	1.10	0.91	0.001*
Capsule thickness (mm)	5.03×10^{-1}	4.56×10^{-4}	3.78×10^{-2}	1.17×10^{-4}	1.91	0.75	0.011*
Whole (embryo+gut) wet mass (g)	1.27×10^{-2}	6.90×10^{-7}	3.08×10^{-4}	1.38×10^{-6}	1.32	0.05	0.640
Whole (embryo+gut) dry mass (g)	2.21×10^{-3}	1.70×10^{-7}	2.25×10^{-5}	1.00×10^{-7}	5.64×10^{-1}	0.38	0.142
Embryo dry mass (g)	1.15×10^{-3}	3.60×10^{-7}	2.84×10^{-5}	1.10×10^{-7}	9.90×10^{-1}	0.68	0.022*
Gut dry mass (g)	1.05×10^{-3}	-2.30×10^{-7}	3.26×10^{-5}	1.40×10^{-7}	1.31	0.35	0.165
Body height (mm)	2.17	-4.84×10^{-4}	3.64×10^{-2}	2.26×10^{-4}	1.46	0.48	0.085
Fin height (mm)	1.79	2.61×10^{-4}	2.67×10^{-2}	9.94×10^{-5}	7.81×10^{-2}	0.58	0.047*
Tail muscle height (mm)	1.17	-3.86×10^{-5}	2.58×10^{-2}	1.78×10^{-4}	1.76	0.00	0.837
Total length (mm)	9.55	2.84×10^{-3}	1.06×10^{-1}	4.29×10^{-4}	7.79	0.90	0.001*
Tail length (mm)	6.44	2.64×10^{-3}	6.73×10^{-2}	2.90×10^{-4}	5.73×10^{-1}	0.94	<0.001*
Snout–vent length (mm)	3.15	3.66×10^{-3}	4.78×10^{-1}	2.11×10^{-4}	1.40	0.38	0.143
Body width (mm)	2.32	-2.42×10^{-4}	2.78×10^{-2}	1.60×10^{-4}	1.03	0.32	0.189
Tail width (mm)	8.58×10^{-1}	-1.23×10^{-4}	1.05×10^{-2}	8.19×10^{-5}	1.08	0.31	0.193
Arcsine square root of % embryo total dry mass	8.07×10^{-1}	1.33×10^{-4}	1.23×10^{-2}	4.58×10^{-5}	1.41	0.63	0.034*
Arcsine square root of % gut of total dry mass	4.79×10^{-1}	-1.32×10^{-4}	1.22×10^{-2}	4.55×10^{-5}	1.42	0.63	0.034*

Linear regression coefficients in the form $y=a+bx$, where y is the variable of concern and x is the water potential, S_a is the standard error of a, S_b is the standard error of b, S_{yx} is the standard error of estimate and R^2 is the coefficient of determination. *Significant regressions (P<0.05).

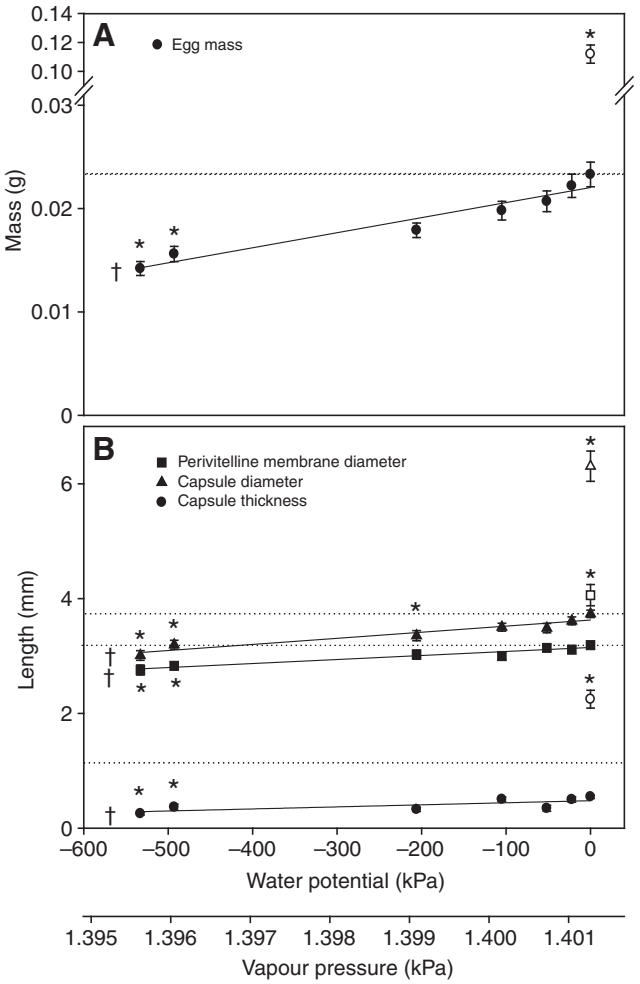

Fig. 1. The relationship between incubation water potential and (A) egg mass, and (B) capsule and perivitelline membrane diameters of stage 26 *G. victoriana* eggs. Error bars are ±1 s.e.m. Values for eggs raised above pure water (vapour water potential, ψ_v=0 kPa; vapour pressure of water, P_{H_2O}=1.4013 kPa) are shown by dotted lines. *Significant difference from values for eggs raised above pure water. †Significant linear regression. Open symbols are the values for eggs raised in shallow pure water (ψ_π=0 kPa). Values are means ±1 s.e.m. *N* values for each treatment are as listed for the variable 'Total length' in Table 3.

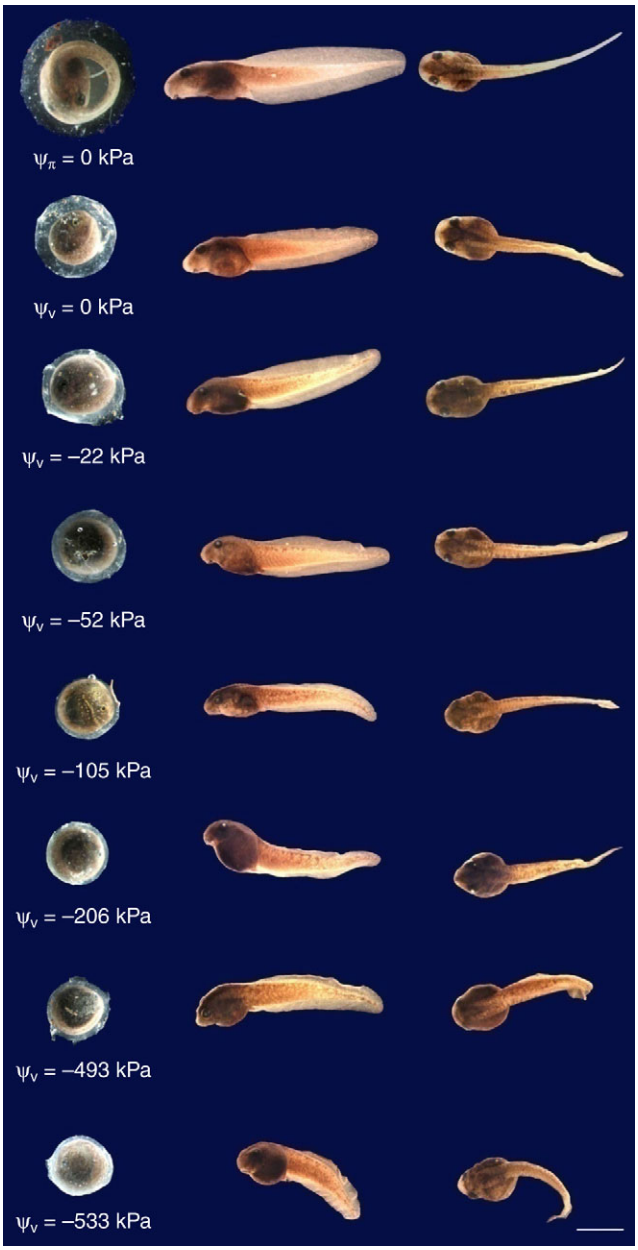

Fig. 2. Examples of unhatched *G. victoriana* embryos and lateral and dorsal views of hatched larvae at Gosner stage 26. The scale bar is 2.5 mm.

(*N*=19) and the mean ovum diameter (embryos <2 days old) was 2.27±0.21 mm (*N*=180 eggs).

Effects of incubation treatments on egg and embryo mass and morphology

Comparison of water vapour treatments

Egg mass decreased linearly with decreasing ψ_v (Table 2; Fig. 1). The diameters of the perivitelline membrane and capsule, and consequently capsule thickness, also decreased with decreasing ψ_v (Table 2; Figs 1 and 2). Eggs from atmospheres below –493 kPa had significantly smaller perivitelline diameters and thinner capsules than control eggs, while eggs in atmospheres below –206 kPa had significantly smaller capsule diameters (Figs 1 and 2).

There were no differences in whole (embryo+gut) wet or whole (embryo+gut) dry mass between the various ψ_v treatments (Table 2; Fig. 3), but dry gut-free embryo mass decreased with decreasing ψ_v. The relative proportion of dry gut to dry body tissue was influenced by ψ_v, with embryos reared in the driest conditions assimilating less yolk than better hydrated embryos. Total embryo length decreased with decreasing ψ_v (Tables 2 and 3; Fig. 2). Stage 26 embryos raised at ψ_v=0 kPa were longer than those embryos raised in atmospheres below ψ_v=–493 kPa. Tail length was shorter with decreasing ψ_v, but snout–vent length was only significantly different between embryos raised at ψ_v=0 and embryos raised at –105 kPa and –493 kPa (Tables 2 and 3; Fig. 2). Body height only differed between embryos from ψ_v=0 kPa and at ψ_v=–533 kPa (Table 3). Fin height decreased with decreasing ψ_v. There was no effect of incubation ψ_v on tail muscle height or tail width (Tables 2 and 3; Fig. 2). Fixed embryo dimensions were 95% of live embryo dimensions (*P*=0.001, Student's *t*-test).

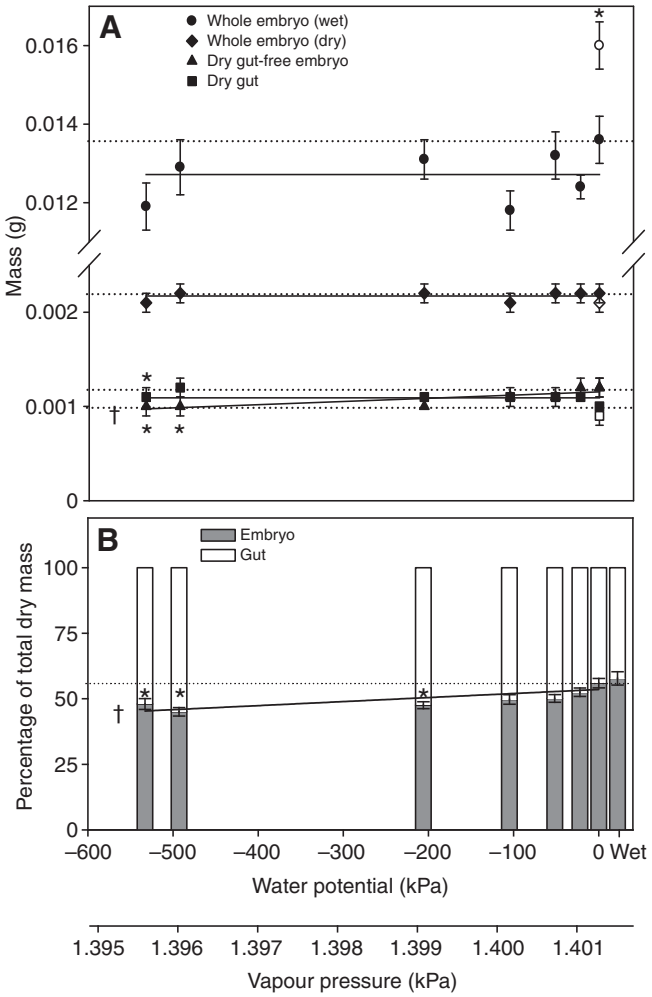

Fig. 3. The relationship between incubation water potential and (A) whole (embryo+gut) embryo wet mass, whole (embryo+gut) embryo dry mass, dry gut-free embryo mass and dry gut mass and (B) the proportion of dry mass in gut and body of stage 26 *G. victoriana* embryos. Values for eggs raised above pure water (ψ_v=0 kPa, P_{H_2O}=1.4013 kPa) are shown by dotted lines. *Significant difference from values for eggs raised above pure water. †Significant linear regression. Open symbols are the values for eggs raised in shallow pure water (ψ_π=0 kPa). Values are means ±1 s.e.m. *N* values for each treatment are as listed for the variable 'Total length' in Table 3.

Comparison of liquid and vapour phase

There were significant differences between *G. victoriana* eggs incubated on a wet substrate (ψ_π=0 kPa) and the control (ψ_v=0 kPa) eggs. Eggs incubated on a wet substrate were 4.8 times heavier than control eggs, the perivitelline membrane and capsule diameters were significantly greater and the jelly capsule was thicker (Fig. 1). Stage 26 embryos raised at ψ_π=0 kPa were 18% heavier and 14% longer than embryos reared at ψ_v=0 kPa (Fig. 3). The greater length of ψ_π=0 kPa embryos was due to both greater snout–vent length and greater tail length. Fin height of embryos raised at ψ_π=0 kPa was 23% higher than for control (ψ_v=0 kPa) embryos (Table 3; Fig. 2).

Osmolality of perivitelline and interstitial fluid

The osmolality of perivitelline fluid measured in unhatched stage 26 embryos was 10±2 mosmol kg^{-1}, which was equivalent to a ψ_π of –24±2 kPa (*N*=16). The osmolality of interstitial fluid from the tails of unhatched stage 26 embryos was 179±2 mosmol kg^{-1} (*N*=3), equivalent to a ψ_π of –399±15 kPa. The osmolality of the heads and residual yolk material was 194±1 mosmol kg^{-1} (*N*=3), equivalent to a ψ_π of –441±2 kPa. On average, the osmolality of the entire embryo (tail+head) was 186±2 mosmol kg^{-1}, equivalent to a ψ_π of –424±5 kPa.

Rate of oxygen consumption

There were no differences in the dry mass-specific rate of oxygen consumption ($\dot{V}_{O_2}$, µl h^{-1} mg^{-1}) for eggs at stage 26 that had been raised at different ψ_v values or between the control and 0 kPa ψ_π embryos (Fig. 4). The pre-hatching stage 26 $\dot{V}_{O_2}$ across all treatments averaged 0.92±0.09 µl h^{-1} mg^{-1}.

Survival rates and hatching success

There was no effect of ψ_v on the percentage of embryos surviving to hatching stage 26 (*P*=0.302) with survival averaging 37.12±7.10% (*N*=42 containers) across the ψ_v treatments. There was also no effect of ψ_v on the number of embryos that were able to hatch when flooded (*P*=0.067), which averaged 74.01±5.23% (*N*=42 containers). The percentage of ψ_v=0 kPa *versus* ψ_π=0 kPa embryos surviving to hatching was not different (*P*=0.17), averaging 39.17±8.21% and 40.00±2.24%, respectively. Similarly, the hatching rates of embryos raised at ψ_v=0 kPa *versus* ψ_π=0 kPa were also not different (*P*=0.994), averaging 74.81±9.96% and 85.40±2.30%, respectively.

Table 3. Morphology of *G. victoriana* embryos incubated to stage 26 at a range of water potentials

Water Potential (kPa)	Total length (mm)	Snout–vent length (mm)	Tail length (mm)	Body height (mm)	Fin height (mm)	Tail muscle height (mm)	Body width (mm)	Tail width (mm)
ψ_π								
0	11.20±0.35* (14)	3.84±0.05* (14)	7.37±0.32 (14)	2.08±0.04 (14)	2.27±0.10* (14)	1.20±0.02 (14)	2.30±0.03 (14)	0.82±0.02 (14)
ψ_v								
0	9.84±0.37 (20)	3.26±0.06 (20)	6.58±0.32 (20)	2.19±0.04 (18)	1.84±0.08 (17)	1.23±0.03 (18)	2.37±0.07 (20)	0.88±0.02 (20)
–22	9.67±0.23 (20)	3.16±0.07 (20)	6.51±0.18 (20)	2.10±0.04 (19)	1.79±0.07 (19)	1.15±0.02 (19)	2.31±0.04 (20)	0.86±0.01 (20)
–52	9.26±0.25 (17)	3.09±0.06 (17)	6.17±0.23 (17)	2.32±0.06 (15)	1.81±0.06 (13)	1.21±0.03 (15)	2.35±0.06 (17)	0.87±0.03 (16)
–105	9.05±0.20 (17)	3.02±0.06* (17)	6.03±0.18 (17)	2.19±0.05 (17)	1.72±0.05 (14)	1.12±0.03 (17)	2.26±0.06 (17)	0.85±0.02 (17)
–206	9.03±0.40 (13)	3.12±0.10 (13)	5.90±0.32 (13)	2.25±0.12 (10)	1.64±0.09 (10)	1.29±0.10 (10)	2.44±0.06 (12)	1.10±0.15* (11)
–493	7.95±0.48* (10)	2.83±0.12* (10)	5.12±0.45* (10)	2.29±0.11 (8)	1.68±0.05 (8)	1.16±0.06 (9)	2.35±0.13 (8)	0.94±0.05 (8)
–533	8.15±0.27* (9)	3.06±0.08 (9)	5.09±0.25* (9)	2.52±0.10* (8)	1.65±0.10 (7)	1.31±0.09 (9)	2.45±0.10 (7)	0.93±0.05 (7)

Values are means ± s.e.m. (*N*); N values vary because some dimensions could not be accurately measured from the digital images. *Significant difference from the control 0 kPa vapour treatment value (*P*<0.05).

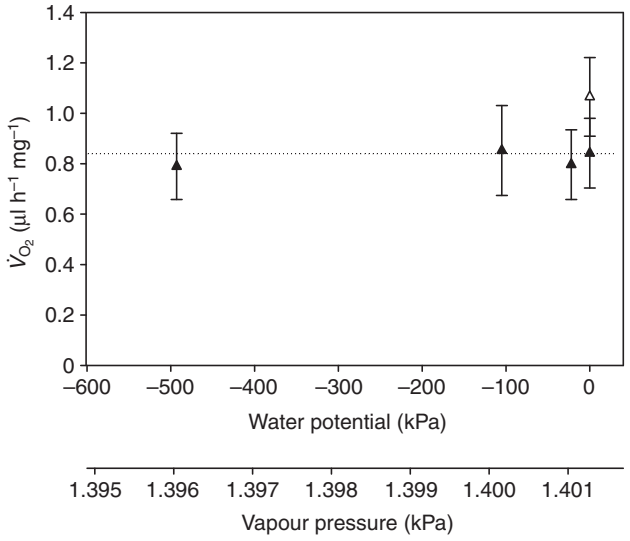

Fig. 4. The relationship between incubation water potential and dry mass-specific rate of oxygen consumption (µl h^{-1} mg^{-1}) of unhatched stage 26 *G. victoriana* embryos. Values for eggs raised above pure water (ψ_v=0 kPa, P_{H_2O}=1.4013 kPa) are shown by the dotted line. Open symbol shows the value for eggs raised in shallow pure water (ψ_π=0 kPa). No statistical significance between means was observed. Values are means ±1 s.e.m. *N* values for each treatment are as listed for the variable 'Total length' in Table 3.

DISCUSSION

Incubation in aqueous *versus* vapour phases

G. victoriana eggs incubated in shallow pure water (ψ_π=0 kPa) were larger and heavier than eggs in saturated vapour (ψ_v=0 kPa; Fig. 1), and the resulting embryos were more hydrated (Fig. 3) and had longer tails with higher fins (Table 3). The two numerically identical water potentials produced two different phenotypes. Why does the egg swell more when placed in shallow pure water (hence hydraulic pressure gradient is negligible) than in 100% RH above the water surface (i.e. when the two conditions have the same water potential)? A likely explanation is that small spatial or temporal gradients in ambient temperature across the incubation chambers prevent equilibrium between the liquid reservoir and the atmosphere around the eggs. The containers were unlikely to be absolutely isothermal, a condition that is further complicated by temperature cycling of the CT room. Small changes in temperature can substantially alter ψ_v above a solution. For example, at a ψ_v of –22 kPa the RH is 99.98% (see Table 1). An equivalent RH can be achieved above distilled water if the air temperature rises approximately 0.003°C, though this would be transient because saturation vapour pressure above the water would eventually be reached. The effect of any temperature differentials across the CT room as a whole will overall have an equal effect on each treatment as the containers were randomised within the CT room. Presumably, the small temperature differentials within the incubation containers add to the differences seen in eggs incubated above liquid water compared with those incubated with access to liquid water, as changes in ambient temperature alter ψ_v only and not ψ_π in pure water. An increase in ambient temperature, or opening of the chambers, will cause a decrease in ψ_v, a situation that will not be reversed when ambient temperature is decreased as RH cannot exceed 100%. The equilibrium was presumably re-established well within 24 h as preliminary testing revealed that a Kimwipe® under similar conditions reached maximal water content within 1 day. Another possibility is that the metabolic heat of the embryo elevates the temperature of the egg a very small amount above the ambient air temperature, hence establishing a water potential gradient where water may be lost as vapour. Fourier's law of heat conduction for a spherical shape using the diameter of the egg and metabolic rate of the embryo reported in this study, and assuming the egg is primarily water (thermal conductivity of water ~0.6 W m^{-1} K^{-1}), reveals that the temperature would be raised by 0.000004°C. This increase in egg temperature would result in a water potential between the egg and the saturated atmosphere at 12°C of –0.0347 kPa; a very small driving force. Debate concerning the effects of temperature on liquid and vapour exchange of water in reptilian eggs has shown the importance of temperature differences on vapour pressure (see Ackerman et al., 1985; Thompson, 1987). Alternatively, the swelling of the egg in liquid water is related to the gel-like properties of the capsule. Cameron and colleagues (Cameron et al., 1990) showed that about 80% of a frog egg's water is osmotically inactive, and further suggested that this water is bound to cell proteins; an idea that supports the notion of the cytoplasm being a gel. The swelling of gels does not simply depend on the difference in ionic concentration between the gel and the medium in which they are placed but on the elasticity of the polymer matrix, with the swollen gel exerting lower restraining forces on the diffusing water molecules (Yasuda et al., 1971; Gunt et al., 2007). Water diffusivity for hydrogels has been shown to be greater when the gels are fully hydrated (liquid phase) compared with the diffusivity of the hydrogel in a vapour phase of equivalent water potential (e.g. 4-fold increase for well-hydrated keratin) (Gunt et al., 2007). Hence, at the same water potential, the egg in liquid will have a higher flux due to a higher diffusivity of water and reach equilibrium earlier. In over-swelling, flux is halted (it has been postulated) because of the increased cross-linking of randomly distributed polymers in the gel, which also act to decrease gel compliance (Kelly et al., 1995). Alternatively, stretch-activated ion channels, which have been found in *Xenopus* oocytes (Yang and Sachs, 1989), may help to limit swelling of the perivitelline space.

Despite potential experimental issues with achieving a ψ_v of 0 kPa, our experiments show the importance for eggs of contacting liquid water in the substrate to allow for swelling of the capsule.

Fitness consequences of development in dehydrating atmospheres

Major components of offspring fitness such as embryonic survival or size can be used as an indication of the effect of the incubation environment on lifetime fitness (Arnold, 1983). In our study, embryos incubated in atmospheres of decreasing ψ_v showed significant declines in whole-egg mass, perivitelline membrane diameter, capsule thickness, dry mass and total length. In particular, tail lengths were significantly shorter for embryos reared in the driest conditions, similar to *B. nimbus* hatching-stage embryos where a decline in total length at lower ψ_π was due to proportionally shorter tails (Mitchell, 2002). Smaller larvae have lower foraging efficiency and may develop more slowly, but in the case of terrestrial embryos, higher rates of yolk assimilation associated with high ψ during incubation may also allow hatchlings to metamorphose earlier and, hence, spend less time in a vulnerable stage of their life cycle (Arnold, 1983). Post-hatching monitoring would be required to determine the optimum ψ_v range for terrestrial development in *G. victoriana*, but our results imply that incubation at ψ_v values below –400 kPa reduces embryonic fitness and is therefore likely to have a detrimental effect on adult fitness. For example, in anurans, larval

size is usually positively correlated with adult size, and larger female *G. victoriana* are more successful breeders (Scroggie, 2001).

Another specific morphological trait likely to influence larval fitness is fin area. This study, like another on *B. nimbus* (Mitchell, 2002), found that fin area was reduced with decreasing water availability (Fig. 2). As tail fins are highly vascularised and hence must be an important respiratory surface, reduced tail fin area could potentially affect rates of metabolism, but we found no effect of incubation ψ_v on the rate of oxygen consumption ($\dot{V}_{O_2}$). At least one study has shown that possession of larger tail fins does not enhance tadpole swimming speed (Van Buskirk and McCollum, 2000a), but larger tail fins may give tadpoles some advantage in escaping predation attempts, as predators are more likely to damage fins rather than tail muscle. For example, larval *Hyla versicolor* are able to lose up to 30% of their tail fin though predatory attempts without affecting swimming performance (Van Buskirk and McCollum, 2000b).

Egg mass decreased as water potential (ψ_v) decreased (Fig. 1), but there was no associated decline in the hydration state of embryos with decreasing ψ_v (Fig. 3), demonstrating the buffering capacity of the jelly capsule in preventing embryonic water loss. The only significant deviation in embryonic water content from the control was for embryos incubated on the wet substrate (ψ_π=0 kPa), whose wet body mass was 18% greater than that of the ψ_v=0 kPa embryos. Dry body mass did not vary between any of the treatments, therefore embryos from the ψ_π=0 kPa treatment simply had a higher water content per unit of dry body mass. However, the proportions of gut and body (gut-free embryo) that constituted the total dry mass varied linearly amongst the treatments (Fig. 3), similar to other studies of anurans and reptiles where 'wetter' embryos converted more yolk into tissue (Packard et al., 1987; Bradford and Seymour, 1988; Miller and Packard, 1992).

We detected no differences in metabolic rate across our treatment groups, a result that differs from studies of *B. nimbus* where $\dot{V}_{O_2}$ of embryos reared at –25 kPa was 72–81% of the $\dot{V}_{O_2}$ of embryos incubated at 0 kPa (Mitchell, 2002). The average $\dot{V}_{O_2}$ across all treatments of $0.92\pm0.09\,\mu l\,h^{-1}\,mg^{-1}$ was very similar to the $0.92\,\mu l\,h^{-1}\,mg^{-1}$ we predicted using a previously published equation (Seymour and Bradford, 1995), where $\dot{V}_{O_2}$ is related to ovum volume, and average $\dot{V}_{O_2}$ is corrected to 12°C using a Q_{10} of 2.5 for *Geocrinia vitellina* (Mitchell, 2001). As $\dot{V}_{O_2}$ is directly related to aerobic metabolism (Mortola and Gautier, 1995), we assumed that embryos in our different treatments were metabolising at the same rate per unit of tissue. However, to have assimilated a greater proportion of yolk into tissue (Fig. 3), the more hydrated embryos must have had a higher mass-specific $\dot{V}_{O_2}$ at some stage in embryonic development.

Survival to hatching stage and hatching success were not influenced by ψ_v across the 0 to –533 kPa range studied, which is comparable to a study of *E. coqui* that found no effect of ψ on embryonic survival between –50 and –550 kPa (Taigen et al., 1984). Studies of other Australian Myobatrachid frogs with terrestrial eggs show different effects of low water potentials; *P. bibronii* have high survival to hatching stage at high ψ (>–50 kPa) but very low survival at ψ less than –400 kPa (Bradford and Seymour, 1988), whereas the survival of *B. nimbus* embryos is severely impaired at –25 kPa (Mitchell, 2002), suggesting a reliance on wet conditions.

Conclusions

Embryonic development of the terrestrial-breeding frog *G. victoriana* was affected by the vapour water potential of the humid atmosphere in which embryos were raised. The difference in morphology was marked, with embryos incubated in atmospheres of decreasing ψ_v showing declines in egg mass, perivitelline membrane diameter, capsule thickness, dry mass, and tail and total length. In the driest conditions (ψ_v=–533 kPa, corresponding to a RH of 99.5% at 12°C), embryos were significantly smaller. Eggs and embryos were also smaller when raised in a saturated atmosphere compared with those raised in contact with pure liquid water, despite these treatments having the same water potential (ψ_v=ψ_π=0 kPa). Presumably, it would be most advantageous for *G. victoriana* to lay its eggs in saturated atmospheres with access to liquid water, a possibility that was observed in some nest site choices in the field. This would ensure larger hatchlings, which would potentially confer an advantage in terms of foraging efficiency.

APPENDIX

Water can move in either the liquid or vapour phase. The driving potential for water movement (i.e. the water potential, ψ) is decided by the free energy (G) of a mass of water, such that water will move spontaneously from high potential to low potential. The total water potential is the sum of a number of component water potentials, including gravitational (ψ_g), pressure (ψ_p), matric (ψ_m), osmotic (ψ_π) and vapour (ψ_v) water potentials. ψ_g is related to the gravitational pull and ψ_p is due to pressure differences from atmospheric pressure, and both are considered negligible in the present study, though turgid eggs would have a positive ψ_p. The potential due to matrix effects (e.g. fluid cohesion and surface tension) is due to adsorption of water onto surfaces. The addition of solutes to water will affect the osmotic potential of the solution. For ideal dilute solutions ψ_π (in kPa) can be calculated from the van 't Hoff equation in terms of solute concentration:

$$\psi_\pi = -ciRT\,, \tag{A1}$$

where c is the molal concentration of the solute, i is the ionisation constant of the solute (e.g. 2 for NaCl; osmolality=ci), R is the gas constant ($8.3144\,J\,K^{-1}\,mol^{-1}$) and T is temperature (K). Hence, as pure water contains no solutes its ψ_π=0.

The vapour water potential (ψ_v, in kPa), the water potential of vapour in air, is also determined from the van 't Hoff relation such that:

$$\psi_v = -\frac{RT\cdot\ln N_w}{V_w}\,, \tag{A2}$$

where V_w is the molal volume of water ($1.8\times10^{-5}\,m^3\,mol^{-1}$) and N_w is the mole fraction of solvent molecules, determined from the following equation:

$$N_w = \frac{n_w}{n_w + n_s}\,, \tag{A3}$$

where n_w is the number of moles of water and n_s is the number of moles of solute in the solution. Raoult's law relates the vapour pressure (P_{H_2O}, in kPa) of equilibrated air above an aqueous solution to the mole fraction of solvent molecules (N_w):

$$P_{H_2O} = P_S N_w\,, \tag{A4}$$

where P_S is the saturation vapour pressure for water (1.40 kPa at 12°C).

Rearranging Eqn A4, it is seen that N_w is equivalent to the relative humidity (RH=P_{H_2O}/P_S=N_w) above the solution. Hence:

$$\psi_v = -\frac{RT\cdot\ln RH}{V_w}\,, \tag{A5}$$

If $n_w \gg n_s$ (i.e. a dilute solution) and the solution is ideal then use can be made of the approximation:

$$N_w = \ln \frac{n_w}{n_w + n_s} \approx \frac{n_s}{n_w + n_s} = N_s \ , \quad \text{(A6)}$$

where N_s is the mole fraction of solute. Rewriting Eqn A6, it can be seen that:

$$\psi_v \approx - \frac{RT \cdot N_s}{V_w} = - ciRT \ . \quad \text{(A7)}$$

Hence, the driving potential for liquid phase flow established by ψ_π in an aqueous solution is the same as that for vapour phase transport established by the ψ_v above the solution.

LIST OF ABBREVIATIONS

c	molal concentration
i	ionisation constant
G	Gibbs' free energy
N_s	mole fraction of solute
N_w	mole fraction of water
n_s	number of moles of solute
n_w	number of moles of water
P_{O_2}	partial pressure of oxygen
P_B	barometric pressure
P_a	vapour pressure of water above an aqueous solution
P_{H_2O}	vapour pressure of water
P_S	saturation vapour pressure above pure water
R	gas constant
RH	relative humidity
T	absolute temperature
t	time
V_w	molal volume of water
$\dot{V}_{O_2}$	rate of oxygen consumption
β_{O_2}	capacitance of water for oxygen
ψ	water potential
ψ_g	gravitational water potential
ψ_m	matric water potential
ψ_p	pressure water potential
ψ_v	vapour water potential
ψ_π	osmotic water potential

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