The influence of hydric environments during egg incubation on embryonic heart rates and offspring phenotypes in a scincid lizard (Lampropholis guichenoti)

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ABSTRACT

Extensive evidence shows that incubation conditions can affect phenotypic traits of hatching reptiles, but the relative importance of thermal versus hydric factors, and the proximate mechanisms by which such factors influence hatching phenotypes, remain unclear for most species. We incubated eggs of an Australian scincid lizard, Lampropholis guichenoti, at four different moisture contents ranging from ~500 to 0 kPa. Drier substrates reduced water uptake of eggs and resulted in smaller hatchlings, but other phenotypic traits (incubation periods, hatching sex, body proportions, running speeds, growth rates post-hatching) were not affected by the hydric environment during incubation. Contrary to our prediction, lower water uptake during incubation (and hence, presumably, more viscous blood) did not affect embryonic heart rates. Thus, in many other squamate species, hatching phenotypes and embryonic developmental rates of L. guichenoti are less sensitive to hydric conditions in the nest than to thermal regimes.

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1. Introduction

The phenotypic traits of individuals are the result of complex interactions between genetic factors and influences of the external environment during development (Via and Lande, 1985). The relative importance of external conditions differs considerably among taxa, and is likely to be especially high in oviparous species, where embryogenesis is not buffered by the maternal system to the same degree as in viviparous species (Deeming, 2004). That sensitivity of hatching phenotypes to incubation conditions will be maximized if degree as in viviparous species (Deeming, 2004). Published studies report a diverse range in the degree to which reptilian embryogenesis is sensitive to the hydric environment, which may reflect the considerable diversity of physiological traits of eggs (such as eggshell structure and water content at oviposition) among living reptile species. For example, in some species there is clear evidence that eggs incubated on wetter substrates produce larger hatchlings that survive better than those from eggs incubated on dry substrates (Packard, 1999; Shine and Brown, 2002; Brown and Shine, 2006). In contrast, moisture content of the substrate has little if any effect on hatching phenotypes in other species (Plummer and Snell, 1988; Lin and Ji, 1998; Ji and Braña, 1999; Flatt et al., 2001; Ji and Du, 2001a,b; Du and Zheng, 2004). Perhaps the most consistent phenotypic effect of the hydric environment involves hatchling body mass: eggs incubated on dry substrates tend to take up less water and (presumably as a result) produce smaller hatchlings in most reptilian species in which hydric effects have been reported (Packard, 1999; Booth, 2002; Du, 2004; Du and Zheng, 2004; Brown and Shine, 2006). Effects of the hydric environment on other fitness-related traits, such as body proportions, locomotor performance and post-hatching growth, have attracted less scientific attention.

Brown and Shine, 2005b) and on norms of reaction for developmental plasticity (Via et al., 1995; Shine, 2004).

The evidence that nest temperatures affect embryonic development and hatching traits is now overwhelming in reptiles, whereas less is known about the effects of hydric variation (Ji and Du, 2001a; Deeming, 2004). Published studies report a diverse range in the degree to which reptilian embryogenesis is sensitive to the hydric environment, which may reflect the considerable diversity of physiological traits of eggs (such as eggshell structure and water content at oviposition) among living reptile species. For example, in some species there is clear evidence that eggs incubated on wetter substrates produce larger hatchlings that survive better than those from eggs incubated on dry substrates (Packard, 1999; Shine and Brown, 2002; Brown and Shine, 2006). In contrast, moisture content of the substrate has little if any effect on hatching phenotypes in other species (Plummer and Snell, 1988; Lin and Ji, 1998; Ji and Braña, 1999; Flatt et al., 2001; Ji and Du, 2001a,b; Du and Zheng, 2004).
Another gap in our understanding involves the proximate mechanisms by which abiotic conditions in the nest influence the developmental biology of reptilian embryos. Most studies have taken a “black box” approach, simply manipulating the embryo’s environment and measuring effects on the hatching (Andrews, 2004). However, several investigators have studied cardiovascular responses to incubation conditions, because the cardiovascular system’s ability to deliver nutrients and oxygen may play a critical role in embryonic development (Birchard and Reiber, 1996; Birchard, 2000). Such studies can provide important insights into embryonic development, but exploring embryonic responses to incubation conditions has been logistically difficult without disturbing embryogenesis. Recent developments in technology allow us to probe the actual mechanisms in more detail by non-invasive monitoring. For example, infrared detectors can monitor heart rate of undisturbed embryos even in tiny lizard eggs, and even at early embryonic stages. The technology has become available only recently, and has been applied to thermal effects on heart rate in lizards (Radder and Shine, 2006), but other issues such as hydric effects on heart rate remain unexplored. Simple physics suggests that lowered water uptake rates to the egg (as occur on dry substrates) may render the blood more viscous and thus, mean that the heart must work harder to pump blood through the embryonic circulation (Packard et al., 2000; Packard and Packard, 2002). Therefore, we predicted that, owing to desiccation, heart rates would be lower for dry-incubated embryos than wet-incubated ones. In the present study, we experimentally incubated scincid lizard eggs at a range of substrate water contents to quantify effects during incubation (on water uptake rates, embryonic heartbeat rates, and incubation periods) and after hatching (on hatching body sizes, body proportions, locomotor abilities and growth rates). Our objectives were to test mechanistic hypotheses concerning hydric effects on reptilian embryos, as outlined above.

2. Materials and methods

2.1. Study species

*Lampropholis guichenoti* is a small (up to 51 mm snout-vent length [SVL]), diurnal, oviparous scincid lizard species, widely distributed in eastern Australia (Cogger, 2000). Female *L. guichenoti* produces multiple clutches of one to four eggs from early November to January (Qualls and Shine, 1997). Previous work on this species has shown that incubation temperatures can significantly affect hatching success, hatching size and locomotor performance (Qualls and Shine, 1998), but has not explored hydric effects.

2.2. Egg collection and incubation

In late October 2007, we captured gravid female *L. guichenoti* by hand from city gardens in Sydney. The females were individually kept in 22×13×7 cm cages filled with moist vermiculite where they could lay eggs. The animals were exposed to a 12 h light: 12 h dark photoperiod and a thermal gradient from 10 to 35 °C for 8 h a day provided by an underfloor heating element; ambient temperature fell to 20 °C overnight. The lizards were fed live crickets twice a week and water was provided *ad libitum*. After the females began to produce eggs in early November, we checked the boxes every day for freshly laid eggs, and obtained a total of 85 eggs from 26 females.

All collected eggs were weighed (±0.001 g) promptly, and individually incubated in 64 mL glass jars filled with vermiculite, which were mixed with different quantity of water to produce four levels of moisture: 0, −2, −200, and −500 kPa, following Lin and Ji (1998) and an empirically derived calibration curve linking water potential to the mass ratio of water to dry vermiculite (M. Thompson, unpubl. data). The eggs were buried in vermiculite and the jars were then placed in an incubator at constant temperatures of 25 °C. The clutch size of *L. guichenoti* (1 to 4 eggs) was too small to enable us to distribute eggs from each clutch over all treatments; we thus assigned eggs from individual clutches randomly to different treatments, to minimize maternal effects. Each jar with moist vermiculite was weighed at the beginning of incubation, and every week throughout incubation we reweighed the jars after removing the eggs. Water was added to compensate for evaporative losses and water taken up by eggs.

2.3. Heart rate of embryos

We measured heart rates of embryos at day 10 and 20 of incubation. The heart rate was detected using a Buddy infrared heart rate monitor (Avian Biotech; Http://www.avianbiotech.com/buddy.htm) placed inside an incubator set at 25 °C. The eggs were brought to incubator temperature by being placed there (still inside their jars) for 1 h. Each egg was then removed and placed individually on the monitor to record heart rate over a two-minute period. We
or without SVL as a covariate. Two-way ANOVAs were employed on growth rates. The effects of hydric environment and sex on locomotor performance were run twice with and without SVL as a covariate on relative tail length, relative head length, mass as a covariate on snout-vent length (SVL), body mass, head length and tail length. Two-way ANCOVAs were conducted on data for morphological traits, with initial egg mass as a covariate. We used the software package STATISTICS 6.0 to conduct one-way ANOVAs and G-tests, to examine the effects of hydric environment on incubation duration, hatching success, and hatching sex. Linear regression was used to examine the relationship between initial egg mass and duration of incubation. We conducted repeated measures ANOVAs to examine temporal changes and hydric effects on heart rates of embryos. Two-way MANCOVA and ANCOVA were employed to test the influence of hydric environment and sex on morphological traits and locomotor performance of hatchlings, with initial egg mass determined after the lizards were placed in an incubator at 25 °C for 2 h. We assessed locomotor ability by chasing the lizards along a 1 m racetrack containing infrared sensors to quantify elapsed times (see Shine and Harlow, 1996 for details).

2.5. Post-hatching growth

Four hatchlings (one each from −500 and −200 kPa treatments and two from −12 kPa treatment) escaped before we conducted our growth experiments. The remaining hatchlings were individually toe-clipped and were raised in two outdoor enclosures with a diameter of 1.5 m. The lizards from each incubation treatment were split equally among the two containers. Each enclosure contained sand, barks, branches and bricks to mimic the habitat usually occupied by hatchlings of this species. The lizards were fed twice a week with live crickets dusted with minerals and vitamins, and water was provided ad libitum.

Weighed egg mass before and after the test, and found that evaporative water loss of eggs was negligible over this short testing period.

2.4. Hatchling morphology and locomotor performance

As soon as hatchlings emerged (from 3 December 2007 to 9 February 2008), they were weighed (±0.001 g) and measured (SVL, tail length, and head length), and their locomotor performance was then weighed after the test. Two-way ANCOVAs were conducted on data for morphological traits, with initial egg mass as a covariate on snout-vent length (SVL), body mass, head length and tail length, and with hatching SVL as a covariate on relative tail length, relative head length. The effects of hydric environment and sex on locomotor performance were run twice with or without SVL as a covariate. Two-way ANOVAs were employed on growth rates.

Table 1

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Hydric environment</th>
<th>Sex</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snout-vent length</td>
<td>F_{3,62} = 2.46, P = 0.07</td>
<td>F_{3,62} = 0.01, P = 0.91</td>
<td>F_{3,62} = 0.38, P = 0.91</td>
</tr>
<tr>
<td>Body mass</td>
<td>F_{3,62} = 3.76, P = 0.02</td>
<td>F_{3,62} = 0.06, P = 0.81</td>
<td>F_{3,62} = 0.49, P = 0.69</td>
</tr>
<tr>
<td>Tail length</td>
<td>F_{3,62} = 1.09, P = 0.32</td>
<td>F_{3,62} = 1.42, P = 0.24</td>
<td>F_{3,62} = 0.65, P = 0.59</td>
</tr>
<tr>
<td>Head length</td>
<td>F_{3,62} = 1.07, P = 0.36</td>
<td>F_{3,62} = 0.20, P = 0.66</td>
<td>F_{3,62} = 0.24, P = 0.87</td>
</tr>
<tr>
<td>Relative tail length</td>
<td>F_{3,62} = 1.36, P = 0.26</td>
<td>F_{3,62} = 3.01, P = 0.08</td>
<td>F_{3,62} = 1.19, P = 0.32</td>
</tr>
<tr>
<td>Relative head length</td>
<td>F_{3,62} = 0.66, P = 0.58</td>
<td>F_{3,62} = 0.48, P = 0.49</td>
<td>F_{3,62} = 0.29, P = 0.83</td>
</tr>
</tbody>
</table>

Locomotor performance

| 25 cm speed             | F_{3,62} = 1.32, P = 0.27 | F_{3,62} = 0.04, P = 0.84 | F_{3,62} = 1.80, P = 0.16 |
| 1 m speed               | F_{3,62} = 1.49, P = 0.23 | F_{3,62} = 0.05, P = 0.82 | F_{3,62} = 1.87, P = 0.14 |

Growth

| Snout-vent length       | F_{3,62} = 0.11, P = 0.95 | F_{3,62} = 4.00, P = 0.049 | F_{3,62} = 0.44, P = 0.72 |
| Body mass               | F_{3,62} = 0.20, P = 0.89 | F_{3,62} = 4.30, P = 0.042 | F_{3,62} = 0.30, P = 0.83 |
| Tail length             | F_{3,62} = 0.28, P = 0.84 | F_{3,62} = 9.36, P = 0.003 | F_{3,62} = 0.07, P = 0.98 |

Two-way ANCOVAs were conducted on data for morphological traits, with initial egg mass as a covariate on snout-vent length (SVL), body mass, head length and tail length, and with hatching SVL as a covariate on relative tail length, relative head length. The effects of hydric environment and sex on locomotor performance were run twice with or without SVL as a covariate. Two-way ANOVAs were employed on growth rates.

Fig. 3. Morphological traits of Lampropholis guichenoti hatchlings from eggs incubated at different moist vermiculate with water potential of −500, −200, −12 and 0 kPa. Data were pooled for the two sexes given the non-significant difference between them, and are expressed as adjusted mean ± SE. Means with different superscripts differ significantly (Tukey’s test).
or hatching size as a covariate. The data on hatching traits of the seven individuals with sex unidentified were not included when we conducted the two-way MANCOVA and ANCOVA. Two-way ANOVAs were conducted to determine whether growth rate post-hatching was influenced by substrate moisture levels during incubation and/or hatching sex. Tukey’s post-hoc multiple-comparisons were used to distinguish among means of significantly affected traits.

3. Results

Hatching success of eggs was high (more than 88% in each treatment), independent of substrate moisture level (G-test, \( G = 0.05, \, df = 3, P = 0.05 \)). Incubation duration was not related to initial egg mass (\( r^2 = 0.0003; \, F_{1,76} = 0.02, P = 0.89 \)), nor was it affected by hydric environments during incubation (\( F_{1,74} = 0.14, P = 0.93 \)). The incubation durations at four treatments of 0, −12, −200, and −500 kPa were 42.2 ± 0.8, 41.9 ± 1.3, 42.7 ± 0.6, and 42.4 ± 0.8 day, respectively. In addition, the hydric environment did not affect hatching sex (G-test, \( G = 0.51, df = 3, P = 0.05 \)), and the overall sex ratio of hatchlings did not depart significantly from the expected 1:1 ratio (G-test, \( G = 0.72, df = 1, P = 0.05 \)).

Heart rates of embryos were similar on day 10 and day 20 of incubation (\( F_{1,35} = 0.08, P = 0.97 \)). Neither hydric environment nor offspring sex had significant impacts on mean heart rates of the embryos (hydric environment–\( F_{1,75} = 0.52, P = 0.67 \); sex–\( F_{1,75} = 1.04, P = 0.31 \); see Fig. 1).

During the first 30 days of incubation, eggs incubated on moister substrates took up more water (i.e., egg mass gain varied among hydric treatments: \( F_{6,68} = 13.41, P = 0.00001; \) see Fig. 2). As a result, hatchlings from moist-substrate incubation were larger (heavier) on average than were hatchlings from drier substrates (Table 1, Fig. 3). However, head and tail length, body shape (relative head and tail length) and locomotor performance were not affected by hydric environments nor by offspring sex (see Table 1, Figs. 3, 4). MANCOVA confirmed that overall, hatching traits were not significantly affected by the hydric environment experienced during incubation (\( F_{18,162} = 1.24, P = 0.24 \)), nor by sex (\( F_{6,57} = 0.55, P = 0.76 \)), nor by an interaction between these factors (\( F_{18,162} = 0.82, P = 0.68 \)).

During the period for which we monitored post-hatching growth, three hatchlings died (two from −500 kPa and one from −200 kPa); the influence of incubation moisture levels on hatching survival thus was not significant (G-test, \( G = 5.10, df = 3, P = 0.05 \)). In outdoor enclosures with sufficient food and water, males grew faster than females, but with growth rates of hatchlings unaffected by substrate moisture levels during incubation (Table 1, Fig. 5).

4. Discussion

Over a wide range of substrate water potentials (from −500 to 0 kPa), hydric environments had no significant impact either on heart rates of embryos, or on most phenotypic traits that we measured at hatching (size, shape, etc) or shortly thereafter (locomotor performance, and post-hatching survival and growth). The sole exception was hatching body mass: reflecting higher rates of water uptake during incubation, eggs kept on moist substrates produced heavier hatchlings than did eggs kept on drier substrates.

Why does dry-substrate incubation often reduce offspring mass at hatching? The causal mechanisms presumably are mediated by reduced moisture availability leading to reduced rates of water uptake by the egg, and thus lower availability of water for embryos (Lin and Ji, 1998; Packard, 1999; Ji and Du, 2001a; Du, 2004). But, how does this lowered water availability affect embryonic development? There may

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**Fig. 4.** The influence of hydric environments during incubation on locomotor performance of hatchlings in the skink, *Lampropholis guichenoti*. Locomotor performance is indicated as mean running speeds over distances of 25 cm and 1 m, respectively. Data were pooled for the two sexes given the non-significant difference between them, and are expressed as adjusted mean ± SE. Numbers above the error bar are sample sizes.

**Fig. 5.** Post-hatching growth of hatchling lizards (*Lampropholis guichenoti*) raised in outdoor enclosures. Growth rates are represented as the mean daily increments of body mass, snout-vent length and tail length. Growth rate was not affected by the hydric environment experienced during the prior incubation period, but differed between the sexes. Data are expressed as mean ± SE. Numbers above or below the error bar are sample sizes.
be multiple pathways, any one or more of which can operate in a single species. The simplest possible mechanism to generate a link between incubation moisture substrate and hatching body mass may be water content, with wet-incubated hatchlings containing a higher proportion of water to dry mass (Zhang and Ji, 2002; Du, 2004; Du and Zheng, 2004). Measures of hatching dry mass in *L. guichenoti* could clarify this issue in future studies. A second possible mechanism is that dry-substrate incubation may result in desiccation of the yolk mass, preventing full utilization of the yolk inside eggs. This phenomenon has been reported in some tropical colubrid snakes (*Brown and Shine, 2006*) and pythons (*Aubret et al., 2003*). This process is evident from the amount of residual yolk left behind in the empty eggshell (*Brown and Shine, 2006*), a variable that could be quantified in future research on our study species.

A third possibility is that embryos with access to more water can support higher rates of metabolism, and thus are able to grow faster than embryos with access to less water (*Miller and Packard, 1992*). For example, lowered water availability inside the egg may increase blood viscosity or decrease blood volume, thereby slowing the rate of embryonic development (*Packard and Packard, 2002*). Under this scenario, we would expect incubation moisture to affect total incubation periods rather than (or as well as) offspring size. Our data do not fit this latter interpretation. First, incubation periods of *L. guichenoti* were unaffected by soil moisture levels. Second, heart rates of embryos from dry-incubated versus wet-incubated eggs were almost identical (*Figs. 1, 2*). We predicted that desiccation would reduce heart rates of dry-incubated embryos; why were we wrong? Direct measurements of hematocrit or blood viscosity in embryos would be useful to clarify this point, but are technically difficult to obtain given the small size of the eggs in this species. Another way forward would be to measure heart sizes in hatchlings: in turtles, embryos incubating in dry conditions have larger hearts than those incubating in wet conditions (*Packard and Packard, 2002*). This phenotypically plastic response may enable embryos to maintain perfusion of peripheral tissues in the face of the reduced volume and increased viscosity of blood (*Packard and Packard, 2002*). A similar phenomenon in our own study species might explain the embryo’s ability to develop rapidly even in the face of limited water availability.

Regardless of the process responsible for a reduced body mass in hatchlings from dry-incubated eggs, the magnitude of the effect on hatching body mass may not be biologically significant. Fitness-related traits such as locomotor performance and growth rates were unaffected by this minor treatment-induced variation in body mass at hatching. Thus, the overall conclusion from our study is that hydric environments during the incubation period have relatively little effect on embryonic development, hatchling traits or rates of post-hatching growth in *L. guichenoti*. These results support and extend the outcomes of studies on other lizard species. Several such studies have reported no effects of the hydric environment on hatchling traits (*Tracy, 1980; Lin and Ji, 1998; Ji and Braña, 1999*). Our study also reveals a lack of significant hydric effects on a range of other traits that have been measured less often (e.g., rates of survival and growth under semi-natural conditions free of pressures from food scarcity or predation) and clarifies one aspect of the proximate effect of dry-substrate incubation on squamate embryos: contrary to our prediction, heart rates of embryos were unaffected by substrate moisture levels. Therefore, our results support the emerging generalization that dry-hatchling phenotype is relatively insensitive to variation in water content of the substrate (*Tracy, 1980; Ji and Braña, 1999; Flatt et al., 2001; Ji and Du, 2004a*).

General statements about the relative importance of thermal versus hydric variation for reptilian embryogenesis are hard to make, because of the enormous diversity of reptiles and the difficulty in evaluating enough traits in enough animals, or directly comparing magnitudes of biologically meaningful variation in such factors in natural nests (*Ackerman and Lott, 2004; Brown and Shine, 2005a*). Certainly, moisture content of the incubation substrate can affect hatching phenotypes in some reptiles (*Packard et al., 1980; Overall, 1994; Phillips and Packard, 1994*). However, a large literature on a diverse range of squamate reptile species shows strong effects of thermal variation on hatching phenotypes, and no such literature exists for hydric effects (*Shine, 2004*). We do not mean to imply that substrate moisture levels are not biologically significant. However, parchment-shelled squamate eggs are able to accumulate water reserves when conditions are suitable (e.g., when the ground is soaked by rainfall) and retain that moisture as a buffer during subsequent dry periods (*Brown and Shine, 2005a*). Thermal variation is intrinsically different, because the embryo and its egg cannot store temperature for later use. Thus, the greater sensitivity of reptilian embryogenesis to thermal than to hydric variation may reflect the organism’s ability to buffer the latter kind of variation more effectively than the former. On a proximate level, then, that insensitivity to hydric variation may reflect the egg’s ability to store water for later use. On an ultimate (adaptive) level, a species like *L. guichenoti* that lays its eggs in shallow nests (*Qualls and Shine, 1998*) likely would expose those eggs to extremely variable hydric environments. Insensitivity of embryonic development and hatchling traits to hydric variation thus may enhance hatching fitness in such conditions. Species that dig deeper (and thus, less hydrically variable) nests, or that have water-resistant (calcareous) eggshells, may not face this selective force and thus, may show greater sensitivity of embryogenesis to experimentally-imposed variation in the hydric environment (*Packard, 1999*). Further studies, on a range of species differing in nest-site types, are needed to test this prediction.

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