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Influence of the Hydric Environment on Water Exchange and Hatchlings of Rigid-Shelled Turtle Eggs

David T. Booth*
Chung Yan Yu

Physiological Ecology Group, School of Integrative Biology, University of Queensland, Brisbane, Queensland 4072, Australia

ABSTRACT

To examine the possible influence of incubation substrate water potential on rigid-shelled chelonian eggs and hatchlings, rigid-shelled eggs from four clutches of Brisbane River turtle (*Emydura signata*) were incubated buried in vermiculite at water potentials of approximately −100, −350, and −850 kPa, and patterns of egg mass change and hatchling attributes were examined. All eggs hatched successfully, and there was no apparent effect of water potential on incubation period, fresh hatchling mass, hatchling water content, or hatchling size. Clutch of origin also had no apparent effect on these attributes when initial egg mass was used as a covariate. However, clutch of origin affected initial egg mass, and clutch of origin and incubation water potential influenced the amount of water exchanged between the eggs and their environment during incubation and the amount of residual yolk found in hatchlings. Substrate water potential has little effect on hatchling outcomes other than the proportion of yolk converted to hatchling tissue during incubation in the rigid-shelled eggs of *E. signata*. It would appear that in general, the substrate water potential during incubation affects the quality of chelonian hatchlings by influencing the amount of yolk converted to hatchling tissue during embryonic development and that this influence is stronger in flexible-shelled eggs than in rigid-shelled eggs.

Introduction

The physical conditions of the incubation environment, such as thermal and hydric regimes, can greatly influence the phenotype of turtle hatchlings (Deeming 2004). The best known and perhaps the most significant in terms of life-history evolution is the phenomenon of temperature-dependent sex de-termination exhibited by many species of turtles, where the incubation temperature at the time of gonad formation determines the sex of the hatchling (Bull 1980). The hydric conditions during incubation can also influence embryonic development because turtle eggs exchange water with their incubation environment (Ackerman et al. 1985; Ackerman 1991; Packard 1991). All turtle eggs investigated to date are capable of absorbing water from the environment, and many of these species have sufficient water in them when laid to complete embryonic development even if a net loss of water occurs during development (Vleck 1991).

Within turtles, two general types of eggshell have been de-scribed (Packard and DeMarco 1991). Most species in the sub-order Cryptodira lay highly permeable flexible-shelled eggs, while most species in the suborder Pleurodira lay less perme-able, relatively rigid-shelled eggs—but even these rigid-shelled eggs are capable of swelling in size when they absorb water from the surrounding environment (Packard 1991; Booth 2002). As a consequence of these differences in eggshell per-meability, water flux across the eggshell of rigid-shelled eggs is generally less than that for flexible-shelled eggs. It appears that the amount and net direction of water flux has little or no effect on embryonic development or hatchling phenotype in species that lay rigid-shelled eggs (Packard et al. 1979, 1981; Leshm and Dmi'el 1986; Packard 1999; Booth 2002). In con-trast, the amount of water exchanged during incubation can influence characteristics such as incubation period, hatchling size, mineral content, amount of residual yolk, and hatchling locomotor performance in species that lay flexible-shelled eggs when these eggs are incubated under dry conditions (Packard 1991, 1999; Miller et al. 1987; Packard and Packard 1989, 1991; Miller 1993; Finkler 1999; Rimkus et al. 2002; Sternadel et al. 2006).

Here we test the hypothesis that the water potential of the substrate that eggs are incubated in affects hatchling attributes of the Brisbane River turtle *Emydura signata*, a species that lays rigid-shelled eggs. We do this by incubating eggs at different water potentials and monitoring the pattern and amount of water exchange during incubation, then measuring resultant hatchling attributes.

Material and Methods

Experimental Protocol

This work was performed under University of Queensland Animal Ethics Approval ZOO/ENT/321/03/URG/H, and eggs used in experiments were collected under Queensland Environmen-tal Protection Agency scientific permit WISP01324003. Four gravid female Brisbane River turtles (*Emydura signata*) were

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collected from a pond on the University of Queensland's St. Lucia campus (27°32′S, 153°00′E) in September 2003, and eggs were harvested from females by application of oxytocin (Booth 2002). Twelve eggs from each clutch were weighed, and a unique number identifying clutch of origin and egg number was written on each shell surface with a graphite pencil.

Two eggs from each clutch were placed into each of six plastic boxes containing 200 g vermiculite and varying amounts of distilled water. Three experimental water potentials were used: −100, −350, and −850 kPa. Each water potential treatment had two replicate boxes. Water potential treatments were set up by adding known amounts of water (1.5, 0.5, and 0.2 g water/g vermiculite) to vermiculite whose characteristic water potential–water content curve was determined by thermocouple psychrometry (Fig. 1). To determine this relationship, varying amounts of water were added to dry vermiculite, and the vermiculite was mixed and sealed in airtight jars for a period of 2 wk. The water potential was then determined using a TRU-PSI sensor (Decagon Devices, Pullman, WA) that had been calibrated with precision sodium chloride solutions (Andraski and Scanlon 2002). Eggs were completely buried (2 cm below the surface) within the vermiculite contained in boxes, boxes were closed with loose-fitting lids that allowed exchange of gases with the surrounding atmosphere, and boxes were placed in an incubator at 30°C. The incubator had an internal fan that continually circulated air throughout the interior of the incubator. Boxes were removed from the incubator and the mass of eggs recorded once per week throughout incubation. While the eggs were out of the box (less than 10 min), any water lost from the vermiculite by absorption into eggs or evaporation was replaced and the vermiculite thoroughly mixed before eggs were placed back into the vermiculite. This procedure limited the variation in the range of water potential for eggs within a particular water potential treatment. Because of the nature of the relationship between water content and water potential of vermiculite (Fig. 1), eggs incubated at −850 kPa are likely to have experienced a larger range of water potentials than eggs incubated at −100 or −350 kPa because of the loss of water from the vermiculite between weekly weighings.

Boxes were placed back into the incubator at a different location each week to counter any effect that thermal gradients within the incubator might have on the experimental outcome. Previous measurements within different parts of the incubator while boxes were in the incubator showed that regional differences in air temperature were less than 0.2°C once the door had been closed for 20 min.

From day 40 of incubation onward, eggs were weighed daily until the shell was pipped, and from day 43 onward, eggs were inspected twice daily to determine time of hatch. After hatching, hatchlings were removed from the incubation box, rinsed with tap water to remove adhering vermiculite, patted dry with paper towels, and placed in a loosely sealed plastic specimen jar labeled with the hatchling's clutch and egg number. The jar was placed back in the incubator for another 48 h to allow full absorption of the yolk sac into the abdomen and to allow the carapace to take on its proper shape (Booth 2002). At the end
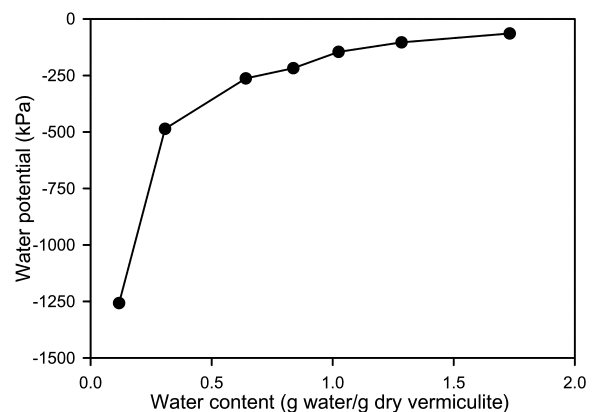

Figure 1. The water content–water potential characteristic curve for the vermiculite used in incubation experiments. Water potential of vermiculite was determined by thermocouple psychrometry.

of this period, hatchlings were weighed and carapace length and width measured with a caliper. Hatchlings were then killed by freezing, thawed, and dissected to separate residual yolk from the carcass. The masses of yolk-free hatchling and residual yolk were recorded. Residual yolks and yolk-free hatchlings were placed in an oven at 60°C and dried to constant masses to determine the dry masses of these components.

Data Analyses

We were interested in the mass of water lost or gained by eggs during incubation, so we followed mass changes by subtracting an egg's mass immediately after it had been laid from its mass on any particular day. Mass changes from days 7 to 42 of incubation were examined in a mixed-model ANCOVA with repeated measurements. In this model, day was the repeated measure, water potential treatment a fixed factor, box a random factor nested within water potential, clutch a random factor across boxes and water potential, and initial egg mass the covariate. Water potential × clutch, day × water potential, and day × clutch interaction terms were also included in this statistical model.

Mixed-model ANOVAs or ANCOVAs where incubation water potential was a fixed factor, box a random factor nested within water potential, clutch a random factor across box, and water potential and initial egg mass the covariate in ANCOVAs, were used to analyze data relating to initial egg mass, amount of water gained or lost during incubation, incubation period, and hatchling parameters. A size index was calculated from the product of hatchling carapace length and carapace width. Because we were also interested in whether the amount of water exchanged during incubation influenced hatchling attributes, we examined these questions using mixed-model ANCOVAs in which clutch was a random factor and initial egg mass and amount of water exchanged during incubation were covariates. An interaction term of clutch × water exchanged was included in these statistical models. Statistical significance was assumed at $P < 0.05$, but we report all probabilities so that readers can

Table 1: Egg and hatchling characteristics of *Emydura signata* eggs incubated at 30°C buried in vermiculite at three water potentials

Parameter	Incubation Water Potential (kPa)			Significance (P)			
	−100	−350	−800	Box	Clutch	Water Potential	Water Potential × Clutch
Initial mass (g)[a]	9.425 ± .122	9.777 ± .112	9.135 ± .158	.478	**.003**	.069	**.004**
Period (d)[a]	47.5 ± .3	47.7 ± .2	47.0 ± .2	.286	.055	.248	.141
Water taken up or lost (g)[b]	1.192 ± .070	.124 ± .063	−.326 ± .062	**.031**	**<.001**	**.005**	.790
Hatchling size index[b]	720 ± 15	712 ± 18	693 ± 16	.910	.234	.602	.644
Hatchling mass (g)[b]	5.558 ± .092	5.326 ± .059	5.510 ± .070	.906	.150	.253	**.063**
Hatchling water content (g)[c]	4.281 ± .078 (77.0%)	4.049 ± .074 (76.0%)	4.263 ± .078 (77.4%)	**.011**	.236	.582	**.022**

Note. Data are means (ANOVA) or least square means ± SE, adjusted for initial egg mass by ANCOVA (adjusted to an initial egg mass of 9.44 g). $N = 16$ for all water potential treatments. Boldface indicates statistical significance at $P < 0.05$.

[a] Mixed-model ANOVA with water potential a fixed factor, box a random factor nested within water potential, and clutch a random factor, with a water potential × clutch interaction term included.

[b] Mixed-model ANCOVA with water potential a fixed factor, box a random factor nested within water potential, clutch a random factor, and initial egg mass as covariate, with a water potential × clutch interaction term included.

[c] Mixed-model ANCOVA with water content (g) as dependent variable, water potential a fixed factor, box a random factor nested within water potential, clutch a random factor, and hatchling dry mass as covariate, with a water potential × clutch interaction term included.

form their own opinion about statistical significance. The software STATISTICA, release 7, was used to perform all statistical analyses.

Results

All eggs hatched successfully. Although there was significant interclutch variation in initial egg mass (Table 1), there was no difference in initial egg mass across water potential treatments (Table 1). Because mass changes associated with exchange of respiratory gases are negligible during embryonic development, virtually all egg mass changes during development are due to the loss or gain of water, so we report any mass changes as water loss/gain. The amount of water lost/gained by eggs varied with days of incubation ($P = 0.034$) and was affected by water potential ($P < 0.001$) and clutch ($P < 0.001$). There was no significant water potential × clutch interaction ($P = 0.982$), but there were significant day × water potential ($P < 0.001$) and day × clutch ($P < 0.001$) interactions, indicating that the pattern of water exchange over time was different for the different water potentials (Fig. 2) and that the pattern of water exchange also varied between clutches. Most eggs at all three water potentials lost a small amount of water (~80 mg) during the first week of incubation, but this was regained during the second week of incubation (Fig. 2). From week 2 of incubation, eggs incubated at −100 kPa gained water throughout incubation, while eggs incubated at −350 kPa had gained a small amount of water by the time of pipping. At −850 kPa, after losing ~80 mg by day 7, eggs slowly regained water, returning to their initial egg mass by day 21, after which they lost water until hatching (Fig. 2).

Incubation period was not influenced by box, water potential, or clutch of origin (Table 1). Box, water potential, and clutch all influenced the prepipping mass of eggs and thus the amount of water exchanged during incubation. Eggs incubated

at −100 kPa gained the most water, eggs incubated at −350 kPa gained a small amount of water, and eggs incubated at −850 kPa lost water (Table 1).

Hatchling mass, size index, and water content were not influenced by incubation water potential or clutch of origin (Table 1). Yolk-free hatchling carcass dry mass and hatchling dry residual yolk mass were influenced by incubation water potential and clutch of origin (Fig. 3).

In the ANCOVAs used to examine whether water exchanged during incubation influenced hatchling attributes (clutch as random factor, initial egg mass and water exchanged during incubation as covariates), the covariate term of water exchanged during incubation was significant for dry residual yolk mass ($P = 0.029$) and dry carcass mass ($P = 0.047$) but not signif-
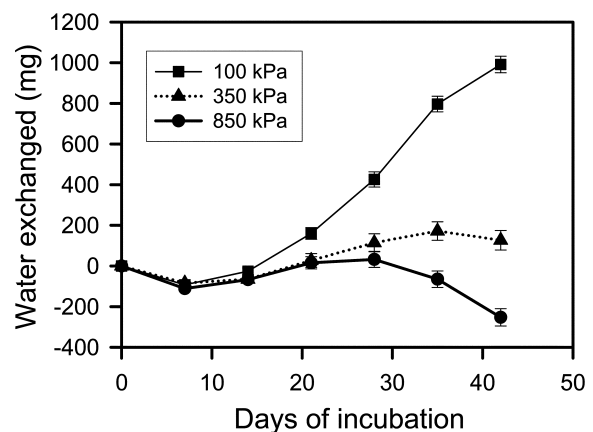

Figure 2. Pattern of water exchange of *Emydura signata* eggs incubated at 30°C buried in vermiculite at three water potentials. Data are least square means ± SE, adjusted for initial egg mass (standardized to 9.44 g) by ANCOVA with initial egg mass as the covariate. $N = 16$ for all three water potential treatments.
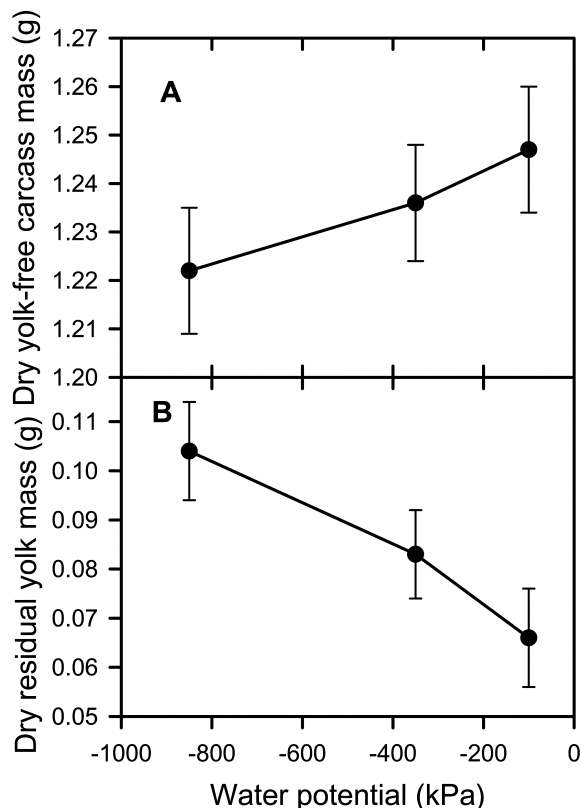
Figure 3. Dry mass of yolk-free hatchlings (*A*) and dry mass of residual yolk (*B*) of *Emydura signata* eggs incubated at 30°C buried in vermiculite at three water potentials. Mixed-model ANCOVA, with water potential as a fixed factor, box a random factor nested within water potential, clutch a random factor, and initial egg mass as covariate, with a water potential × clutch interaction term, indicated significant effects of water potential and clutch in both cases. Data are least square means ± SE, adjusted for initial egg mass (standardized to 9.44 g) by ANCOVA. $N = 16$ for each water potential. Post hoc Tukey HSD tests indicated that both dry yolk–free hatchling mass and dry residual yolk masses were significantly different ($P < 0.01$) in hatchlings emerging from eggs incubated at −100 and −850 kPa.

icant ($P > 0.05$) for hatchling mass, hatchling size, or hatchling water content. These analyses indicated that only dry residual yolk and dry hatchling mass were influenced by water exchange during incubation.

Discussion

Water Exchange by Eggs during Incubation

Water can be exchanged across the eggshell as water vapor (Tracy et al. 1978; Ackerman et al. 1985; Thompson 1987; Ackerman 1991; Packard 1991) and liquid water if a liquid bridge is maintained between the substrate surrounding the egg and the egg contents (Tracy et al. 1978; Ackerman et al. 1985; Thompson 1987; Ackerman 1991; Packard 1991). In eggs incubated in vermiculite, as in the current study, it is likely that water is exchanged entirely by water vapor (Ackerman et al. 1985; Ackerman 1991). Whether eggs gain or lose water during incubation depends on the difference in water vapor pressure

between the egg contents and the substrate surrounding the egg. If the water vapor pressure inside the egg is less than that of the surrounding substrate, there will be a tendency for the egg to gain water, but if the water vapor pressure inside the egg is greater than that in the substrate, then the egg will tend to lose water.

The substrate water potential influenced the total amount and direction of water movement across the eggshell, with all eggs incubated at −100 kPa gaining water and all eggs incubated at −850 kPa losing water before pipping. These general trends have been previously reported in both rigid-shelled and pliable-shelled turtle eggs (see Packard 1999 for review). However, the rates and direction of water flux changed in consistent ways during the course of our experiments.

All eggs lost water during the first week of incubation, despite the fact that eggs incubated at −100 and −350 kPa were exposed, at least in theory, to an environment that should have favored water uptake from the substrate. When rigid-shelled eggs are laid, their pores are filled with fluid, but once they are laid, water is removed from these pores either by evaporation to the surrounding substrate or by reabsorption via the outgrowth of extraembryonic membranes that make contact with the inside surface of the eggshell to form the white patch that characteristically appears during early development of rigid-shelled reptile eggs (Thompson 1985). The loss of water from the eggshell via evaporation may explain why eggs from all water potential treatments lost mass during the first week of incubation. Another possible explanation for the loss of water during this period could be that eggs lost significant amounts of water during the 10–20 min that elapsed between the initial weighing of eggs and the time when they were buried in vermiculite.

Water gained by eggs incubated at −100 and −350 kPa can be explained by the water vapor difference between egg fluids and the incubation substrate because the estimated water vapor pressure inside a turtle egg (−800 kPa; Ackerman 1991) was predicted to be lower than the surrounding substrate. The larger difference in water vapor between embryonic fluid and the substrate surrounding eggs at −100 kPa compared to those incubated at −350 kPa could be explained by the fact that eggs incubated at −100 kPa absorbed more water than those incubated at −350 kPa. An increase in eggshell water vapor conductance (caused by part of the inner eggshell adjacent to the chorioallantois being dissolved in order to provide calcium for the ossifying skeleton) during the later half of incubation would be one possible explanation for why the rate of water gain by eggs increased in eggs incubated at −100 kPa during this period. A possible reason for the loss of water between days 35 and 42 at −350 kPa could be that a small increase in egg temperature during this time, due to the metabolic heating by the growing embryo, increased the water vapor pressure inside the egg to a level above that in the vermiculite surrounding the eggs.

However, the pattern of water exchange in eggs incubated at −850 kPa is more difficult to explain. Because the estimated water potential inside the egg (−800 kPa) was greater than that

of the substrate, we anticipated that water would be lost from the egg throughout incubation. However, eggs gained a small amount of water between days 7 and 28, and we have no satisfactory explanation for this observation. However, eggs did lose water between days 28 and 42 of incubation, as anticipated. The increase in rate of water loss toward the end of incubation was most likely caused by a combination of a slight increase in egg temperature and an increase in eggshell water vapor conductance.

Clutch of origin affected both initial egg mass and the amount and pattern of water exchange during incubation. Significant interclutch variation in egg size is a common feature of freshwater turtle populations (Booth 1998), and variation in egg size may contribute to interclutch variation in water exchange because egg size directly affects the surface area over which water exchange between egg contents and surrounding substrate occurs. Interclutch differences in eggshell porosity and thus water vapor conductance are likely, and differences in osmolarity of egg contents that affect the water vapor pressure inside the egg may also occur. Interclutch differences in one or both of these attributes can explain interclutch differences in the amount of water exchanged during incubation at the same substrate water potential. If interclutch differences in eggshell water vapor conductance were the major reason for interclutch differences in water exchange, one would expect clutches with higher eggshell conductances to gain water faster at -100 kPa but also to lose water faster at -850 kPa. In our experiment, the clutch that gained the most water at -100 kPa also lost the least water at -850 kPa. Likewise, the clutch that gained the least water at -100 kPa also lost the most water at -850 kPa. These observations suggest that it is unlikely that differences in eggshell conductance are responsible for the interclutch differences in water exchange we observed. However, our observation would be consistent with the hypothesis that interclutch differences in water exchange were due to differences in egg content osmolarity if the clutch that took up the most water also had the greatest osmolarity.

Influence of Hydric Environment on Hatchling Attributes

As has been previously reported for other turtle species that lay rigid-shelled eggs (Packard et al. 1979, 1981; Leshm and Dmi'el 1986; Packard 1999; Booth 2002), the water potential of the incubating environment had no influence on the incubation period, live hatchling mass, body dimensions, or water content of *Emydura signata*. This is in stark contrast to results from flexible-shelled freshwater turtle eggs, where the water potential of the incubating medium has a strong influence on these hatchling attributes (reviewed in Packard 1999), although it has been argued that this phenomenon occurs only when water uptake from the substrate is limited or negative (Rimkus et al. 2002). However, water potential of the incubating medium did influence the amount of water exchanged during incubation and the distribution of dry matter within *E. signata* hatchlings, with more yolk solids being transformed into embryonic tissue during development in wetter incubation conditions (Fig. 3).

The finding that the covariate "water exchanged during incubation" was significant suggests that the influence of substrate water potential on dry residual yolk in the hatchling is mediated by the amount of water exchanged during incubation. Exactly the same trend is seen in flexible-shelled eggs of freshwater turtles (see Packard 1999 for review), although this phenomenon appears to be much more distinctive in species that lay flexible-shelled eggs. Rimkus et al. (2002) have suggested that embryo water content may be regulated so that development in dry conditions where water stress may occur retards embryonic growth, resulting in less raw yolk material being converted into hatchling tissue during the incubation period. This could explain why less yolk was converted to hatchling tissue in our experiments.

In summary, rigid-shelled turtle eggs have patterns of water uptake and loss similar to those of flexible-shelled freshwater turtle eggs (i.e., they take up water from the environment at water potentials greater than approximately -350 kPa and lose water to the environment at water potentials less than approximately -350 kPa) when incubated at similar water potentials (although the magnitude of the water flux is considerably less in rigid-shelled eggs). However, unlike in turtles that lay flexible-shelled eggs, in turtles laying rigid-shelled eggs, the incubation period and live hatchling mass and dimensions are unaffected by the water potential of the incubating medium. However, the amount of yolk material mobilized into the hatchling tissue during incubation appears to be influenced by the water potential of the incubation medium in both rigid-shelled and flexible-shelled eggs. The amount of water exchanged between the egg and its substrate is probably the underlining explanation for the differences in yolk utilization in hatchlings from eggs incubated at different substrate water potentials.

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