Osmotic Balance in the Eggs of the Turtle *Chelodina rugosa* during Developmental Arrest under Water

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ABSTRACT

The tropical Australian turtle *Chelodina rugosa* normally lays its hard-shelled eggs in mud, under shallow freshwater, during the monsoon season. The eggs undergo developmental arrest until the water recedes and oxygen is able to diffuse into the embryo. This period of arrest can exceed 12 wk without embryonic mortality. To understand how the eggs avoid osmotic absorption of water leading to shell rupture and embryonic death, this study investigates the solute concentrations and volumes of the albumen and yolk compartments during submergence in distilled water. The albumen loses considerable sodium through the shell, particularly during the first week, and its osmotic concentration drops from 234 mmol/kg at laying to about 23 mmol/kg. Meanwhile, water from the albumen slowly moves through the vitelline membrane into the yolk compartment, which enlarges at a constant rate until it approaches the inside of the shell at about 22 wk. Osmotic uptake dilutes yolk solutes, decreasing the osmotic concentration from 281 mmol/kg at laying to 132 mmol/kg at 157 d. Loss of embryonic viability is associated with contact of the vitelline membrane with the inside of the shell. The principal adaptation of this species for protracted developmental arrest under water is a vitelline membrane of such low permeability to water that the expansion of the yolk compartment occurs about 10 times more slowly than in other chelonians.

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Introduction

Exchange of water between a reptilian egg and its environment depends on several factors, among which the difference in water potential between the environment and egg contents is of primary importance (Packard and Packard 1988). If the egg contents have a lower water potential than the environment, the egg must take up water, either as a liquid (Thompson 1987) or as water vapor (Ackerman et al. 1985) or both. Flexible-shelled eggs of some reptiles often take up water from the environment, and the egg swells. In some reptilian species, this is a requirement for proper hydration of the embryo, but in hard-shelled eggs of other groups, sufficient water is invested in the eggs at laying, and osmotic uptake is not required. Nevertheless, if hard-shelled eggs of some turtles and crocodilians are incubated on wet substrates, they may absorb water and crack the shells (Packard and Packard 1988). Cracking is not necessarily detrimental, especially if the underlying shell membranes remain intact. If the egg does not rupture, the embryo develops normally (Webb et al. 1977; Packard and Packard 1988). In some turtles, namely, kinosternids and batagurines, however, osmotic uptake can eventually rupture the shell membranes and cause leakage of egg fluids, ingress of pathogens, and death of the embryo (Ewert 1985).

The potential problem of too much water is particularly relevant in the case of the Australian northern snake-necked turtle, *Chelodina rugosa,* which lays its eggs in excavations in mud under shallow freshwater in seasonally flooded billabongs (ox-bow lakes) in the Northern Territory (Kennett et al. 1993*a*). The eggs undergo a period of developmental arrest that lasts until the dry season, when the water recedes and the mud dries. Developmental arrest presumably occurs because of anoxia, but when oxygen reaches the eggs, the embryos commence normal development (Kennett et al. 1993*b*). The period of submergence can exceed 12 wk, which places the egg under a protracted osmotic challenge. Measurements of conductivity of floodplain billabongs in the Magela Creek system of the Northern Territory indicate osmotic concentrations below 0.4 mmol/kg in the flood season, rising to a maximum of about 3.4 mmol/kg at the end of the dry season (Hart and McKelvie 1986). By comparison, the osmotic concentration of a fresh turtle egg would be expected to be in the region of 200–300 mmol/kg.

Kennett et al. (1993*b*) provide explanations for the prevention of egg swelling and rupture: (1) the "ink bottle" effect that prevents liquid water from invading the eggshell pores

of several avian eggs (Sotherland et al. 1984), (2) a possible impermeability or hydrophobic adaptation of the shell membranes, and (3) loss of osmolytes from the egg contents, reducing the osmotic gradient. The first mechanism appears unlikely because it relies on an air-water interface that could not form in eggs laid underwater. Regardless of the shape of the pores, water bridges must occur through the pores in both the shell and shell membranes and connect the environmental water with that in the interior. Even if water bridges were somehow avoided, water vapor would move down a gradient in water potential into the egg. The second mechanism likewise entails an air-water interface or suffers from the fact that membranes that are impermeable to water are also impermeable to respiratory gas. Also, shell membranes of reptilian eggs are mats of proteinaceous fibres that have pores much too large to impede water movement (Ewert 1985; Packard and Packard 1988). The third suggestion, that osmotic materials diffuse out from the egg, is highly likely. Although this would reduce the osmotic gradient, it could not eliminate the problem, especially in eggs incubated for over 12 wk in freshwater.

Another possibility is that the vitelline membrane that surrounds the yolk compartment is the main barrier to water uptake. In other reptilian eggs, cracking of the shell and rupture of the shell membranes occurs when the water in the albumen is absorbed into the yolk compartment and the vitelline membrane begins to push against the inside of the shell membranes (Ewert 1985; Packard and Packard 1988). If the uptake of water into the yolk were delayed in *C. rugosa*, the egg could withstand immersion longer. We designed this study to test this hypothesis by determining the time course of yolk expansion during arrested development in distilled water. We also measured the solute concentrations in the yolk and albumen compartments to determine the changes in osmotic gradients, and we measured how much material was lost from the eggs into the external water.

Material and Methods

Series 1

Two clutches of *Chelodina rugosa* eggs were obtained from females following hormonal injection (Syntocin; 10 IU/kg body mass). The turtles were left in water in large plastic bins. Female 1 released her eggs within 4 h on July 9, 1990, and female 2 released part of her clutch after the initial dose and the remainder after a 5 IU/kg dose on the next day. The eggs were removed from the water, weighed within 2 min, and placed in distilled water. On July 17, the eggs were packed in soaking wet cotton wool and flown to Adelaide. One egg from clutch 1 was destroyed for pilot anatomical investigations. On July 18, 16 eggs from clutch 1 and seven eggs from clutch 2 were sealed individually in new 70-mL plastic specimen jars with 44 mL of double-distilled water. They were incubated at 30°C until the last sample was taken, 157 d after laying.

Series 2

Because it was discovered that most of the ions left the eggs of series 1 during the 9-d interval before the measurements began, we obtained two additional clutches (clutch 3 and 4) from two females on March 26, 1996, to investigate the pattern of changes during the first 9 d in water. Five eggs (two from clutch 3 and three from clutch 4) were removed immediately from the water after they were laid and sealed in dry specimen jars with dry cotton wool; these represented fresh eggs. Thirteen other eggs (six from clutch 3 and seven from clutch 4) were placed individually in specimen jars with about 44 mL of double-distilled water, and all eggs were flown to Adelaide for analysis and further treatment. Upon arrival, they were kept at 30°C for up to 9 d.

Analysis

At selected times during the immersion period, the concentrations of cations (Na^+, K^+, Ca^{++}, Mg^{++}) appearing in the water were analyzed with a GBC 904 atomic absorption spectrophotometer. The mean concentrations of these ions in the fresh double-distilled water were not significantly different from 0. Cation efflux rates were calculated from concentrations in the jars with eggs and a blank containing distilled water only. After each reading, the water in each jar was discarded and replaced with 44 mL of fresh distilled water.

Three to six eggs were selected for analysis of contents from time to time. All eggs measured during the first 9 d came from clutches 3 and 4; the rest came from clutch 1. They were removed from the water, wiped dry, inspected for cracking of the shell, and opened with a diamond saw around the equator. The contents were removed, and the albumen was carefully scraped from the yolk membrane with a blunt spatula and sucked into a syringe. The wet egg shell, albumen, and yolk were weighed to within 1 mg. Mass loss because of sawing and evaporation during this procedure averaged 1.6% of the whole egg mass and was ignored. The albumen spontaneously separated into a thin clear fraction and a thick translucent fraction, and the yolk granules settled to the bottom of the yolk compartment, leaving a clear supernatant. Triplicate samples of the thin albumen and supernatant yolk were analysed immediately with a Wescor 5100C vapor pressure osmometer. Because the readings of this osmometer depart from linearity at osmotic concentrations less than 100 mmol/kg, and many of the albumen samples were less than this, these readings were corrected according to a separate standard line drawn between values given by the instrument from triple-distilled water and a calibration standard of 100 mmol/kg. Other samples of albumen and yolk were appropriately diluted with triple-distilled water,

and the concentrations of cations (Na^+, K^+, Ca^{++}, Mg^{++}) were analysed with the atomic absorption spectrophotometer.

Statistical Analyses

Statistics provided are means and 95% confidence intervals (CI). Groups are compared with two-tailed *t*-tests, and coefficients of determination (r^2) are given for model 1 (least squares) regressions.

Results

The four clutches contained 17, 7, 8, and 10 eggs, respectively. It was not determined whether these numbers represented all of each female's eggs because no X-ray examinations were performed. The mean egg mass from four clutches was 14.66 g (±2.16 CI, $n = 4$). The eggs were not weighed sequentially during immersion but were assumed to be of constant mass because of the rigidity of the shell. A *t*-test showed that the mean mass (14.58 g) of six uncracked eggs from clutch 1 that had been in water for less than 39 d was not significantly different from six eggs (two of which had cracked shells) from clutch 1 that had been submerged for 157 d (14.57 g). No eggs were cracked when observed at 39 d immersion. Five eggs from clutch 1 showed some cracking at 88 d. No further cracking occurred after this time, so that at 157 d, 11 eggs remained uncracked, including all of the eggs in clutch 2.

Wet mass of the shell (including shell membranes) of three clutches averaged 15.4% (±2.8 CI) of the total egg mass (14.66 g), leaving 12.40 g of egg contents. Of the contents, the albumen accounted for 34.8%, and the yolk was 63.3%, in five fresh eggs (Table 1). The percentage of albumen decreased slowly as the percentage of yolk increased during immersion in water (Fig. 1). The equation for percentage albumen content (A) on time (t) is $A = -0.200t + 33.7$ ($r^2 = 0.96$), and for percentage yolk content (Y) it is $Y = 0.206t + 64.3$ ($r^2 = 0.95$).

The total osmotic concentration of the yolk was significantly greater than that of the albumen in five fresh eggs (paired *t*-test, $P = 0.001$; Table 1). The yolk was higher in sodium concentration ($P = 0.03$), but the albumen was higher in potassium ($P < 0.001$) and magnesium ($P < 0.001$). There was no significant difference in calcium concentration ($P = 0.07$). Twice the sum of the measured cations was not significantly different from the measured osmotic concentration, either in the albumen ($P = 0.24$) or in the yolk ($P = 0.86$), providing a rough indication that the measured cations and their complementary anions account for most of the osmotic concentration. The mean fresh egg contained 1.02 mmol of solutes in the albumen and 1.24 mmol in the yolk (Table 1). The amount of solute in the yolk was calculated assuming that (1) the fresh yolk consisted of a solid fraction of protein and lipid having negligible osmotic activity, (2) the initial solid fraction was 44% (mean of eight chelonian species [Ewert 1979, Table 8]), and (3) the initial yolk was 63.3% of the egg contents. Thus the fresh egg contents included 27.8% of yolk solids. The total amount of dissolved osmolyte in the yolk compartment was calculated by multiplying the measured osmotic concentration (mmol/kg) by the mass of yolk water (= yolk mass − 0.278 [egg mass − shell mass]).

As the yolk volume increased, its osmotic concentration decreased significantly (Fig. 1). A least squares regression of yolk osmotic concentration (Ψ) and time (t) was $\Psi = -0.86t + 258$ ($r^2 = 0.90$). After 157 d, mean solute concentration was 132.1 mmol/kg (±6.8 CI). The decrease appeared to result largely from uptake of water rather than loss of osmolytes. The amount of yolk solutes decreased from 1.24 mmol (±0.19 CI) to 1.12 mmol (±0.08 CI) after immersion for 157 d. However, the slope of the regression of total yolk osmolyte on time was not significantly different from 0 ($r^2 = 0.14$). Osmotic

Table 1: Composition and solute concentrations of the contents of five fresh eggs of *Chelodina rugosa*

		Albumen		Yolk	
	Units	Mean	CI[a]	Mean	CI[a]
Fraction of contents	. . .	.348	.023	.633	.028
Sodium	mmol/kg	92.5	19.2	132.7	10.3
Potassium	mmol/kg	20.2	2.2	3.7	1.9
Calcium	mmol/kg	1.0	.5	1.7	.1
Magnesium	mmol/kg	15.0	1.2	4.3	.6
Total cations	mmol/kg	128.5	19.8	142.3	12.6
Total cations × 2	mmol/kg	256.9	39.7	284.6	25.1
Osmotic	mmol/kg	234.2	7.4	281.6	6.4
Total solutes	mmol	1.02	.20	1.24[b]	.19

[a] 95% confidence interval.

[b] Assuming 44% yolk solids.

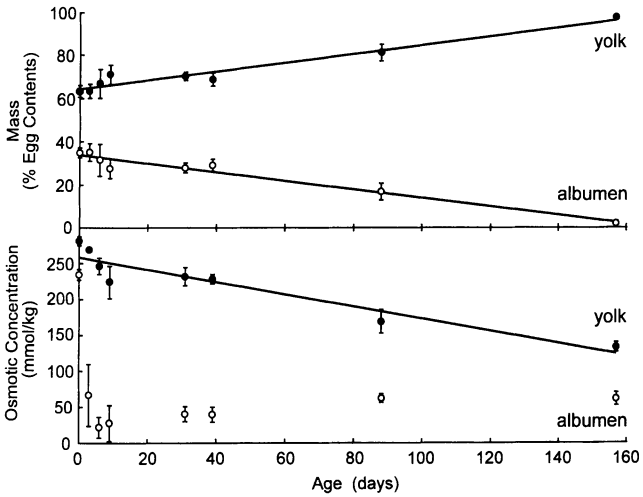

Figure 1. Mass of yolk and albumen as a fraction of total egg contents (*top*) and osmotic concentration (*bottom*) in *Chelodina rugosa* eggs during immersion in water. All points are means and 95% confidence intervals. The points within the first 9 d are from clutches 3 and 4; those after 9 d are from clutch 1. Model 1 linear regressions are also presented (see text for statistics).

concentration of the albumen began at 234.2 mmol/kg (a total of 1.02 mmol initially present), but after the first 6 d of immersion, it dropped to values averaging about 23 mmol/kg (Fig. 1). After 157 d, the mean solute concentration of the albumen was 28.4 mmol/kg (±16.2 CI), and only 0.0053 mmol (±0.0052 CI) remained in 1.46 g of albumen. A total of 1.14 mmol was lost from the egg, 1.02 mmol from the albumen and 0.12 mmol from the yolk.

Sodium efflux from the eggs into the distilled water was measured in four clutches; clutches 3 and 4 were measured during the first 9 d and clutches 1 and 2 were measured thereafter. Rates of sodium efflux decreased with time (Fig. 2). A power equation satisfactorily described the relationship between sodium efflux (Y) and time (t): $Y = 115.8t^{-1.0675}$ ($r^2 = 0.88$). The total sodium lost into the distilled water during the 157-d immersion was 0.56 mmol, as integrated from this equation. During the first 9 d, about 0.12 mmol of potassium and 0.06 mmol of magnesium were lost. After 9 d, the rates of potassium and magnesium loss were assumed to be negligible. The constancy of calcium loss (Fig. 2) suggested that calcium came primarily from the shell, rather than from the contents. Therefore the total cation loss from the egg contents was estimated to be 0.74 mmol. Assuming anions accompanied these cations, the total loss was 1.48 mmol. This figure is about 19% higher than that calculated by analysis of egg contents (1.24 mmol), but the difference is within the confidence limits of the initial egg contents.

Discussion

This study demonstrates that the eggs of *Chelodina rugosa* do not reach osmotic equilibrium with a freshwater environment during arrested development. There is fluid contact between the albumen and the environment, and solutes from the albumen diffuse out through the shell. Loss of solutes occurs mainly during the first weeks underwater (Fig. 2), resulting in low osmotic concentrations of the albumen (Fig. 1). Meanwhile, there is a progressive uptake of water from the albumen into the yolk compartment that continues for about 22 wk, after which practically no albumen remains (Fig. 1).

A constant osmotic concentration gradient of approximately 23 mmol/kg prevails between the environment and the albumen after most of the ions have left the egg. This presumably results from the presence of large proteins that cannot pass through the limiting membrane on the inside of the shell membranes (Lillywhite and Ackerman 1984; Dumont and Brummett 1985). At 30°C, this concentration should cause an internal osmotic pressure of about 58 kPa (= 435 mmHg) (Milburn 1979). We have no direct measurement of this pressure, but it is likely that the shell can withstand it. The flexible-shelled eggs of the colubrid snake *Elaphe obsoleta* rupture at pressures above 57 kPa (Lillywhite and Ackerman 1984), but calcareous shells would be expected to withstand greater pressures. The osmotic concentration of albumen in fresh *C. rugosa* eggs was 234 mmol/kg, which would be expected to produce a pressure of about 600 kPa. Such calculations are of doubtful value, however, because solute concentration would result in an equal osmotic pressure only if the membrane separating the albumen from the environment were impermeable to all solutes and equilibrium were attained. This is clearly not the situation in *C. rugosa* eggs that leak small ions, and therefore the maximum internal pressure in *C. rugosa* eggs is likely to be much less

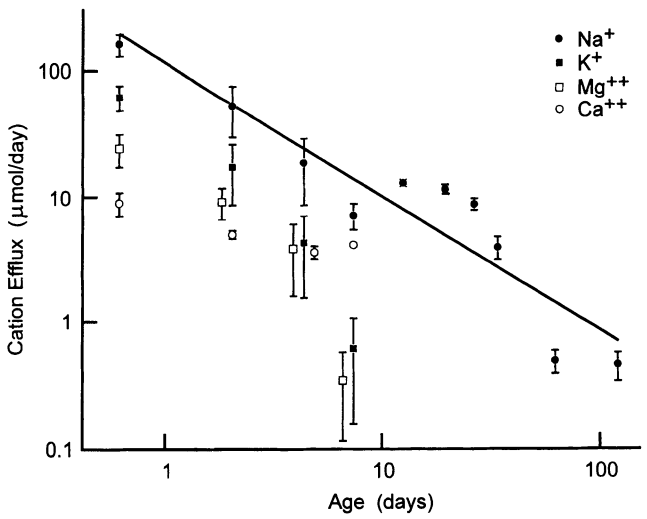

Figure 2. Rates of cation efflux (µmol/d) from eggs of *Chelodina rugosa* during immersion in distilled water. Means and 95% confidence intervals are plotted at the center of each measurement period. The data within the first 9 d are from clutches 3 and 4; those after 9 d are from clutches 1 and 2. A power regression is presented for sodium efflux (see text for statistics).

than 600 kPa. It is interesting that the osmotic pressure of fresh eggs of the turtle *Chrysemys picta* has been estimated to be 335 kPa (Packard et al. 1981) and 444 kPa (Packard et al. 1983). The albumen of fresh chicken and turkey eggs exerts an osmotic pressure of 654 kPa and 553 kPa, respectively (Tullett and Board 1976).

Conditions in the albumen stabilize within 1 wk, but the yolk compartment continues to change for over 20 wk (Fig. 1). The decrease in osmotic concentration of the yolk compartment and its increase in volume indicate that the total amount of active solute in the yolk compartment changes little over this period. Because the total volume of the egg is essentially constant, the expansion of the yolk occurs largely by uptake of water from the albumen. The failure of the albumen to increase in osmotic concentration as it loses water over this period (Fig. 1) results from continued slow loss of small solutes to the environment.

It is clear that the main characteristic of *C. rugosa* eggs that is correlated with long submergence in water is a very low water permeability of the vitelline membrane surrounding the yolk compartment. The vitelline membrane is produced in the female's oviduct and is distinct from the yolk sac membranes that grow from the embryo later in development. There are a few morphological studies of this structure (Dumont and Brummett 1985), but we know nothing of its functional characteristics, especially under conditions of anoxic developmental arrest. However, we do know that the yolk compartment normally expands during development in reptiles (Ewert 1979, 1985; Packard and Packard 1988). In fact, it begins to expand even before the egg is laid (Agassiz 1857). Turtle eggs with either flexible or hard shells possess considerable albumen, but practically all of the water in the albumen is absorbed into the yolk compartment during the first 1–2 wk of normal embryonic development (Ewert 1979; Packard et al. 1981; Packard and Packard 1988). Only in rare cases does an appreciable amount of albumen persist throughout incubation (Ewert 1979).

Expansion of the yolk compartment also occurs during arrested development in oviducally retained turtle eggs, and Ewert (1979) has suggested that the embryos begin to lose viability when the expansion is complete and no albumen remains. Turtle embryos that survive release from long oviducal arrest often show abnormal development (Ewert 1985). It is possible that the pressure of the expanding yolk against the inside of the rigid shell eliminates sufficient space in which the embryo can develop. If so, the slow expansion of the yolk in *C. rugosa* would be essential for survival for long periods of submersion.

The difference in tolerance to submersion between *C. rugosa* and the congener *Chelodina longicollis* is striking. Embryos of *C. longicollis* do not survive submersion of even 2 wk (Kennett et al. 1993*b*). Moreover, hatchability of fresh eggs of *Trionyx muticus* declines greatly after 2 d in water and reaches 0 after 15 d (Plummer 1976). Although we do not know the cause of mortality, the coincidence between the survival limit of about 2 wk in *C. longicollis* and *T. muticus* and the normal time course of yolk expansion of 1–2 wk in other turtles is consistent with the idea that viability declines when the vitelline membrane begins to contact the shell. It is doubtful that cracking of the shell or rupture of the shell membranes is directly responsible for mortality in *C. rugosa* eggs. Only five of 23 eggs from clutches 1 and 2 that were immersed for more than 2 wk showed some cracking of the shell, and none ruptured. Swelling of *C. longicollis* eggs is not correlated with mortality; obvious swelling of the egg to the point of eggshell cracking does not appear until more than 2 wk after the embryos become nonviable (Kennett et al. 1993*b*).

It is of interest that so few *C. rugosa* eggs cracked, including none in clutch 2, even after 22 wk in distilled water. At this time, there was practically no albumen remaining, and it is possible that the vitelline membrane was beginning to push against the inside of the shell. The potential pressure of 332 kPa (>3 atm) would be exerted by the yolk because its osmotic concentration averaged 132 mmol/kg (Fig. 1). We suggest that either the shells can withstand such internal pressure or the yolk had not completely expanded.

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