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The effects of nest environment on calcium mobilization by leatherback turtle embryos (*Dermochelys coriacea*) during development

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Abstract

We investigated the effect of sand moisture content and sand temperature on developmental success and the mobilization of calcium during development using laboratory incubated eggs ($n = 251$) collected from leatherbacks nesting at Parque Nacional Marino Las Baulas, Costa Rica. Calcium concentrations of egg components [eggshell, yolk plus albumen (Y + A) and embryo] changed significantly through incubation for both viable and undeveloped eggs. In developed eggs, eggshell calcium content decreased 42.9% by day 60 of incubation. The Y + A calcium decreased by 20.8% until the last quarter of incubation, and then increased to 0.99% above initial Y + A calcium concentrations just prior to hatching. In undeveloped eggs, eggshell calcium content decreased by 25.7%, with the rate of decrease slowing significantly beyond day 30 of incubation. In contrast, Y + A calcium increased steadily through the 60-day incubation period. Embryos incorporated a higher proportion of calcium when incubated at a lower sand moisture content (5% H_2O > 12% H_2O) and at lower sand temperatures (28.5°C, 29.5°C > 31.0°C). The total wet mass of freshly oviposited eggs was negatively correlated with calcium concentration per gram of eggshell ($r = -0.569$; $P < 0.001$). Thus, each yolked egg, regardless of initial wet mass, had an average of 1.23 g (± 0.43 g) of calcium per egg (Mean egg mass: 76.24 ± 1.21 g). Both developmental success (24.1%) and hatching success (7.4%) of laboratory-incubated eggs were dependent to a greater extent on temperature than on moisture, with an increase in mortality as sand temperature increased. For natural nests on Playa Grande, developmental success (37.4%) and hatching success (19.8%) were similar in magnitude to the results obtained from the laboratory. The recent ENSO (El Niño Southern Oscillation) event and increased tidal activity may be responsible for the high embryonic mortality measured during the 1997–1998 nesting season.

Keywords: Calcium mobilization; *Dermochelys coriacea*; Eggs; Developmental success; Embryos; Sea turtles; Costa Rica

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1. Introduction

Nest location and the properties of substrate type (i.e. soil moisture tension, thermal dynamics, permeability and porosity, etc.) have been correlated with egg developmental success in environments where water absorption is in excess of water loss (Tracy et al., 1978; Packard et al., 1985; Packard and Packard, 1987). The degree of egg hydration influences ion and nutrient exchange between the chorioallantoic membrane (CAM) of the eggshell, the yolk reserve and the embryo (Packard and Packard, 1986, 1989; Packard, 1994a,b). Ions such as calcium, phosphorus and magnesium are required for the ossification of the embryonic turtle skeleton and are obtained from the maternally derived yolk reserve and other egg components (Simkiss, 1962; Bustard et al., 1969). The leatherback embryo obtains approximately 75% of its calcium from the eggshell (Bustard et al., 1969). Simkiss (1962) suggested some calcium may be obtained externally, as he reported for magnesium. It was this finding that warranted the need for further investigation of external ionic sources for embryonic development (i.e. calcium), in addition to the influence of environmental factors on ionic exchange.

Environmental determinants of egg-substrate ion and water exchange have been extensively studied and reviewed (e.g. Ackerman, 1997; Packard and Clark, 1996). Packard and Packard (1989) demonstrated that snapping turtle (*Chelydra serpentina*) eggs incubated on wet and dry substrates showed marked differences in calcium and phosphorus mobilization from yolk reserves and the eggshell. A similar hydration effect occurs in the eggs of ornate box turtles, *Terrapene ornata* (Packard and Packard, 1988). In general, turtle eggs incubated in wetter substrates (-150 kPa water potential) mobilized calcium and phosphorus more efficiently and at faster rates than on drier substrates (-950 kPa water potential). The thermodynamic conditions found within the nest environment also have a significant impact on the developmental success of the turtle embryo, determining such factors as embryonic mortality and the sexual differentiation of hatchlings (Bustard and Greenham, 1968; Ackerman, 1980, 1997; Whitmore and Dutton, 1985; Spotila et al., 1987; Packard and Packard, 1989; Binckley et al., 1998). Incubation temperature is negatively and positively correlated with incubation period and embryonic mortality, respectively, within physiological limits (Ackerman, 1997; Binckley et al., 1998).

Calcium is mobilized from the egg contents via a transepithelial mechanism utilizing the ectodermal cells of the CAM (Tuan et al., 1991; Akins and Tuan, 1993a,b; Packard, 1994a,b). The process is thought to be unidirectional, suggesting that the net flux of calcium leaving the eggshell is equal to the net flux of calcium internalized by the embryo and its associated compartments (Tuan et al., 1991; Akins and Tuan, 1993b). Transport of calcium is also dependent on the hydration within the inner eggshell membrane (ISM), requiring at least a thin molecular layer of water (Packard and Packard, 1989; Tuan et al., 1991). This is consistent with the effects of substrate water potential on the rates of growth and calcium internalization by aquatic turtle embryos (Packard and Packard, 1986, 1989).

It is unclear why the degree of hydration within a given substrate influences the rate of calcium uptake by turtle embryos. Simkiss (1962) and Bustard et al. (1969) determined the sources of calcium and ions utilized by leatherback embryos, but the effects of hydric conditions and temperature of the nesting environment on the rate of ionic mobilization have not been examined. Questions regarding the function and composition of yolkless eggs (a common component of *Dermochelys coriacea* clutches) also require investigation. Although hypothesized as a mediator of gas exchange and thermal regulation within the nest (Rostal et al., 1996; Steyermark et al., 1996), there are no data on their composition and relation to viable eggs.

The purpose of this study was to investigate the effects of the natural and artificial nest environment on the success of development and the physiological mobilization of calcium by leatherback turtle embryos. The rates of calcium mobilization, the overall developmental and hatching success (as compared with calcium concentrations), and the relative impact on survival (i.e. embryonic mortality) during different stages of development were determined for different treatments of sand moisture tension and sand temperature in a laboratory setting. Sources of embryo calcium were investigated, whether obtained solely from an endogenous source or absorbed from the surrounding sand/absorbed water. Calcium concentrations from both viable

and yolkless components were determined and examined for similarities and/or differences in relation to one another. The results were also assessed for possible significance in the context of conservation and management.

2. Materials and methods

2.1. Study site

We selected a nesting beach where *D. coriacea* nests in large numbers and the collection of eggs would impact less than 1% of the total number of eggs deposited. Eggs were collected from two adjacent leatherback nesting beaches, Playa Grande (10°18.6 N, 085°50.30 W–10°20.32 N, 085°51.23 W) and Playa Ventanas (10°20.38 N, 085°51.21 W–10°20.76 N, 085°51.65 W), Parque Nacional Marino Las Baulas, Costa Rica. The nesting substrate (i.e. sand type) found on these beaches is medium grained, composed of silica sand and shell material (J.R. Spotila, unpublished data).

2.2. Experimental design and sample collection

We randomly collected freshly oviposited eggs from 'doomed' nests (nests susceptible to tidal inundation or erosion) throughout the nesting season, with the number sampled depending on the conditions of the nesting event (location in tide-washed zone, stage of nesting, etc.). We recorded nesting parameters during egg collection following methods outlined by Steyermark et al. (1996).

We determined the calcium content of eggs at the time of oviposition (day 0) and at different stages during incubation. We transported each sampling group to the laboratory (approx. 3 km from the beach) in new, double-ply trash bags containing a sand sample collected from the nest chamber. A total of 281 yolked and 17 yolkless eggs were collected from randomly selected females, 7–20 eggs per clutch (27 clutches); 251 yolked eggs were artificially incubated at varying degrees of sand temperature and moisture, and the remaining 30 eggs sacrificed immediately after collection. We also collected live and dead hatchlings (40 total) and 'hatched' eggshells (40) from Playa Grande and Playa Ventanas in early January through February 11, 1998; live hatchlings (from the beach and the incubators) were euthanized by rapid freezing, and dried (85 ± 5°C) to a constant mass. We cleaned, weighed (± 0.005 g) and labeled eggs with a wax pencil. Eggs collected for calcium determination at day 0 were sacrificed immediately upon arrival at the laboratory, separated into their individual components (eggshell with adhering membranes and yolk plus albumen (Y + A) or albumen only for yolkless eggs) and dried to constant mass. We then placed individual components into a new plastic bag, and immediately placed that bag into a larger air-tight, plastic bag containing 500 g of Drierite (W.A. Hammond Drierite Co.). We stored all samples at room temperature under standard atmospheric conditions for laboratory analyses.

Eggs were incubated in Styrofoam, thermal airflow Hova-Bators (G.Q.F. Mfg. Co., Savannah, GA). We filled 12 Hova-Bators with 9.5 kg each of sifted Playa Grande sand (approx. one-half their volume) collected at beach depths of 50–60 cm. Hova-Bators were segregated by treatments of sand temperature (°C) and sand moisture content (% H_2O), one treatment per incubator. Treatments ($3 \times 2 \times 3$) comprised three sand temperatures (28.5°C, 29.5°C — pivotal sex-determining temperature, 31.0°C) at two different sand moistures (5% H_2O, 12% H_2O) and three different times during development (day 30, day 45 and day 60) six treatments × one replicate. We randomly distributed eggs from each clutch among the incubators and placed eggs at a uniform depth in the sand with the top surface exposed (approx. 2 cm in diameter). This simulated the air spaces between eggs within the natural nest, which were important for gas exchange (Paladino, personal observation). We sampled eggs from each treatment at day 30 (approx. halfway point of incubation), day 45 (start of last quarter of development), and time of hatching (day 60).

We measured incubator temperatures using 24-gauge copper-constantan (Cu–Cn) thermocouples placed in the sand at the center of each Hova-Bator. We recorded temperatures once or twice daily using a BAT-12 thermocouple reader (± 0.05°C). Incubator temperatures were maintained to within ± 0.5°C of the set temperature. The desired percent sand moisture for each Hova-Bator was determined by gravimetric analysis. Calibration curves were used to determine the desired moisture percentage, derived from empir-

ical data. If sand moisture content fell below the desired percentage, we added distilled water (volume calculated from calibration curves) with a spray bottle to the incubator to ensure an even moisture distribution.

Eggs sampled from the Hova-Bators were treated similarly to beach eggs sacrificed at the time of oviposition. We carefully removed, cleaned, and weighed each egg. We freed embryos from the attached extra-embryonic membranes and separated the remaining egg contents into their individual components [eggshell with adhering membranes — CAM plus inner shell membrane (ISM), Y + A and embryo]. We cleaned hatchlings and their respective eggshells of any adhering sand and weighed them separately. Hatchlings were euthanized by rapid freezing and later dissected free of their yolksac. We dried egg components from laboratory incubated eggs and fresh eggs and stored them for analysis as previously described.

2.3. Developmental success and hatching success

We determined developmental and hatching success of eggs in the Hova-Bators and in nests on Playa Grande and Playa Ventanas, using a modification of the method employed by Leslie et al. (1996). We excavated nests ($n = 22$) shortly after hatching (1–5 days), and recorded the total number of yolked (includes 'hatched' eggshells) and yolkless eggs. We dissected unhatched eggs in situ and staged them using the criteria in Table 1, a modification of the criteria outlined by Leslie et al. (1996) and Binckley et al. (1998). We applied the same staging method to eggs sampled from the incubators. We determined developmental success as the percentage of eggs showing visible signs of development from the total number of yolked eggs. Hatching success was estimated as the percentage of 'hatched' eggs (the number of broken eggshells or total number of live and dead emerged hatchlings) plus the pipped eggs (both live and dead hatchlings), divided by the total number of yolked eggs.

2.4. Determination of calcium content

We determined calcium content of egg and hatchling samples using atomic absorption spectrophotometry (Perkin-Elmer Model 107) following the method of Columbia and Hill (1997). Samples were first digested with 70% perchloric acid using a non-flame heat source. Digestion was rendered complete once gas was observed to flux against the sides of the beaker (15–20 min) and/or no suspended particulate was present. Samples were then removed, cooled, diluted with distilled water to a known volume, and further diluted for analysis by atomic absorption spectrophotometry.

2.5. Statistical analyses

Statistical analysis for the effects of sand temperature and sand moisture content, time of incubation, and sample type on calcium content was performed using SigmaStat and SPSS statistical software. A $3 \times 2 \times 2$ design was used to test for significant differences between calcium concentrations of egg components (eggshell, Y + A and embryo): duration of incubation (day 30 and day

Table 1
Categories and definitions used for the staging of eggs (modified from Steyermark et al., 1994)

Category	Stage of development	Definition
U	Undeveloped	No visible signs of development
EED	Early embryonic death	White spot on shell or dead unpigmented embryo < 20 mm in length
MED	Middle embryonic death	Dead pigmented or unpigmented embryo ≥ 20 mm and < 40 mm in length
LED	Late embryonic death	Dead pigmented embryo > 40 mm in length
P	Pipped	Penetrated egg containing live or dead unemerged hatchling
H	Hatched[a]	Live or dead hatchling (in nest and/or emerged[b])

[a] Hatchling that has successfully emerged from its eggshell.
[b] Hatchling that has reached the sand surface.

60; day 45 excluded), sand moisture content (5% H_2O and 12% H_2O), and sand temperature (28.5°C, 29.5°C and 31.0°C). We performed combined-between treatment analysis using a univariate analysis of variance (ANOVA) with a Type IV sums of squares calculation for missing data cells (95% C.I.). *t*-Tests and Mann–Whitney rank sum tests tested for significant differences in calcium concentrations between egg components. A one-way ANOVA and the Tukey test tested for significant differences in calcium concentrations between sampling periods. Chi-square analysis ($\chi^2 = 0.05$) determined significant differences in developmental and hatching success for laboratory eggs incubated in different temperature and moisture treatments. Simple linear regression tested for correlation between initial egg mass (day 0; wet mass) and eggshell and Y + A calcium concentrations. We assumed significance at $P = 0.05$. Results are shown as the mean ± one standard error (S.E.M.).

3. Results

3.1. Distribution of calcium during incubation

Freshly oviposited yolked eggs (day 0) contained an average of 1.23 g (±0.43 g) calcium per egg. Mean calcium concentrations for dry mass of eggshell and Y + A were 412.92 mg g^{-1} and 7.01 mg g^{-1}, respectively, suggesting that at the start of incubation the eggshell contained approximately 90% of the egg's total calcium content (Table 2). Calcium concentration in eggshells of yolkless eggs (day 0) was 66.2% of that contained in eggshells from yolked eggs (Table 2, $P < 0.001$). The calcium content of albumen alone, sampled from yolkless eggs, was 1.59 mg g^{-1}. This amount could not be compared with the albumen calcium content from yolked eggs, as the yolk and albumen (Y + A) were not separated during analysis.

Table 2
Comparisons of calcium concentrations (Ca^{2+} mg g^{-1}) for egg components sampled from fresh, non-incubated yolked and yolkless eggs

Egg type	Sample type	Ca^{2+} (mg g^{-1}) ± S.E.M.
Yolked	Shell	412.92 ± 7.98 ($n = 30$)
	Y + A	7.01 ± 0.28 ($n = 30$)
Yolkless	Shell	273.40 ± 18.72 ($n = 15$)
	A only	1.59 ± 0.38 ($n = 15$)

Calcium concentrations contained within all egg components changed significantly through time. In developed eggs ($n_{beach} = 40$; $n_{Hova\text{-}bators} = 26$), mean eggshell calcium concentrations declined 42.9% (approx. 177.13 mg g^{-1}) by day 60, with the majority of change occurring after day 45 (Fig. 1a). Y + A (in hatchlings: yolksac with remnants of albumen) calcium in artificially incubated eggs declined by 20.8% (7.01 mg g^{-1}) until day 45, and then increased 21.5% (from 5.56 to 7.08 mg g^{-1}, respectively) from day 45 until the time of hatching and time of absorption of the yolksac (2 ± 1 days) into the abdomen (Fig. 1b). Hatchlings ($n_{beach} = 40$; $n_{Hova\text{-}Bators} = 5$) gained an average of 27.29 mg g^{-1} (±1.75 mg g^{-1}) of calcium by the time of hatching (approx. 941.8 mg Ca per hatchling), 76.6% of the mean total calcium content of the egg (Fig. 2).

Undeveloped eggs also showed significant changes in egg component calcium concentrations through incubation (P_{Shell}, $P_{Y+A} = < 0.001$, *t*-test) (Fig. 1a,b). Eggshell calcium decreased by 25.7% (306.91 mg g^{-1}), with the rate of decline slowing in the second half of incubation (Fig. 1a). Calcium content of Y + A, in contrast to eggshell calcium, increased steadily to 11.92 mg g^{-1}, with a gradual increase in slope as the incubation period approached day 60 (Fig. 1b). Because yolkless eggs were not artificially incubated or collected from hatched nests beyond day 60, changes in the calcium content of albumen and eggshell through the incubation period are not known.

Mean initial mass (wet) of freshly oviposited eggs was 76.24 g (±1.21 g, $n = 30$). When dried at constant temperature, the eggshell weighed 2.10 g (±0.04 g) and Y + A 12.70 g (±0.15 g). Eggshell calcium concentrations of fresh eggs were negatively correlated with the total wet mass of the egg ($r = -0.569$; $P \leq 0.001$). The Y + A calcium concentrations, however, showed no statistically significant correlation with initial egg mass. Means for both the expected and observed eggshell calcium concentrations were comparable for different times of incubation, with the exception that the expected mean values tend to be slightly less than the observed. No correlations were found between hatchling mass/size and calcium concentrations of eggshell and yolksac.

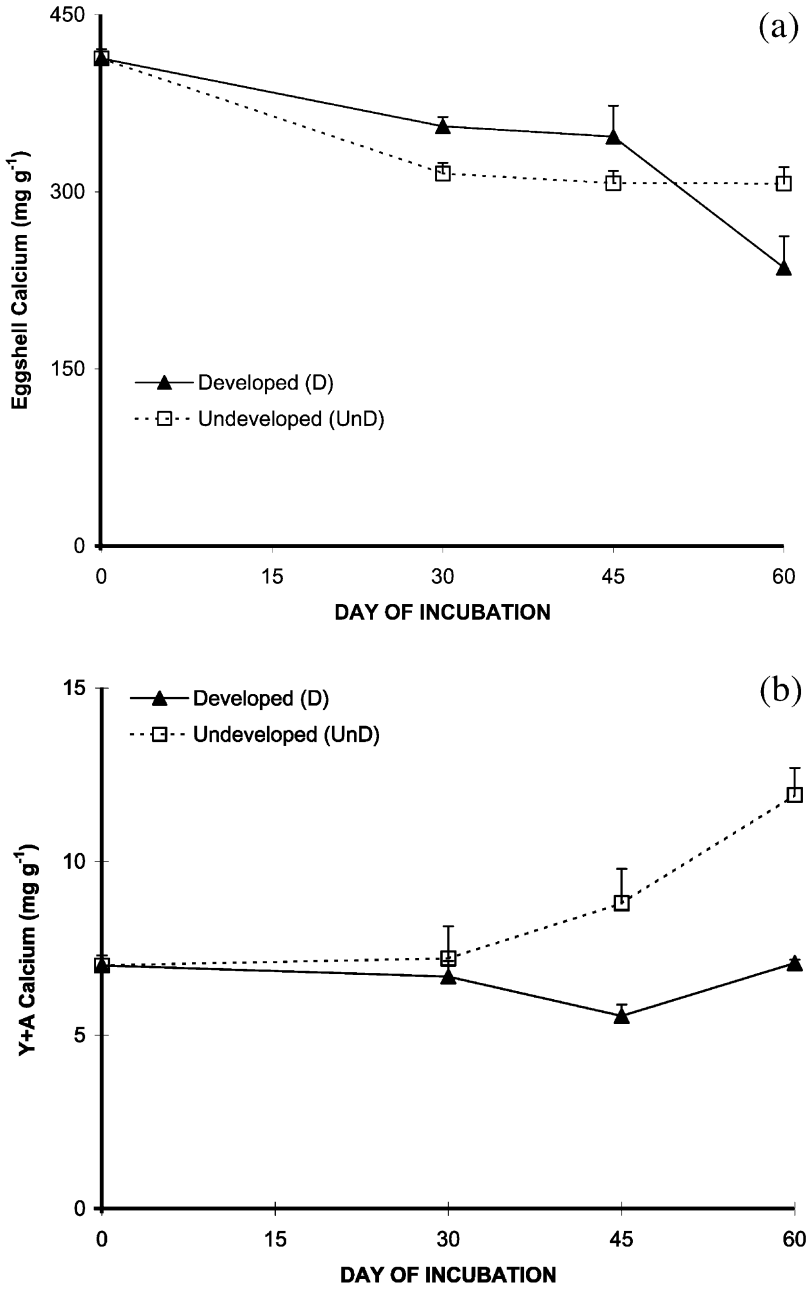

Fig. 1. (a) Change in eggshell calcium concentrations (mg g^{-1}) for developed (D) vs. undeveloped (UnD) eggs. Error bars shown represent ± 1 standard error. (b) Change in yolk plus albumen calcium concentrations (mg g^{-1}) for developed (D) vs. undeveloped (UnD) eggs. Error bars shown represent ± 1 standard error.

3.2. Rate of calcium mobilization in relation to treatment

The rate of calcium uptake differed significantly with incubation time for treatments of moisture ($P = 0.004$) and temperature ($P = 0.015$). Embryos incorporated calcium faster when incubated at lower sand moisture (5% H_2O > 12% H_2O) and lower temperature (28.5°C, 29.5°C > 31.0°C) (Table 3). Sample sizes for all treatments were small ($N_{total} = 24$; not including day 45 embryos shown in Fig. 2), with some treatments yielding only one or no embryos (missing data cells) for examination (no samples yielded

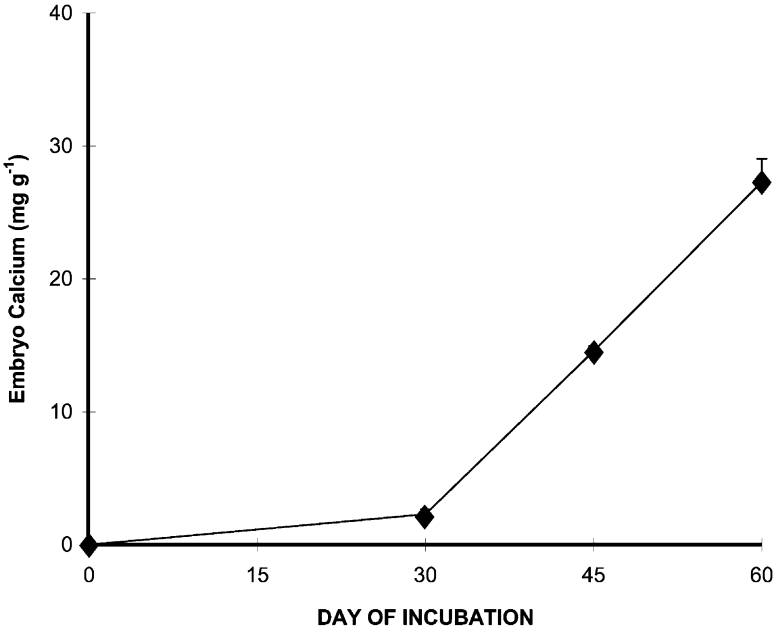

Fig. 2. Change in embryonic calcium concentrations (mg g^{-1}). Error bars shown represent ± 1 standard error.

from the 12% H_2O–31.0°C treatment). The two-way interaction between moisture and temperature and the three-way interactions between time, moisture and temperature were not statistically significant.

3.3. Developmental success and hatching success

Developmental success and hatching success for nests oviposited on Playa Grande after Octo-

Table 3
Estimated marginal means for sample time vs. moisture and sample time vs. temperature

Test ($N_{total} = 24$)	Sample time (day)	Moisture (% H_2O)	Temperature (°C)	Mean Ca^{2+} (mg g^{-1}) ± S.E.M.
Time vs. moisture	30	5	–	2.96 ± 0.58 ($n = 13$)
		12	–	1.49 ± 0.68 ($n = 6$)
	60	5	–	29.15 ± 0.87 ($n = 3$)
		12	–	24.50[a] ± 1.07 ($n = 2$)
Time vs. temperature	30	–	28.5	2.18 ± 0.81 ($n = 9$)
		–	29.5	2.13 ± 0.81 ($n = 8$)
		–	31.0	2.36 ± 1.07 ($n = 2$)
	60	–	28.5	27.60 ± 1.07 ($n = 2$)
		–	29.5	28.38 ± 1.07 ($n = 2$)
		–	31.0	24.50[a] ± 1.51 ($n = 1$)
Time	30	–	–	2.22 ± 0.45 ($n = 19$)
	60	–	–	27.29[a] ± 0.67 ($n = 5$)
Moisture	–	5	–	16.05 ± 0.52 ($n = 16$)
	–	12	–	10.69 ± 0.59 ($n = 8$)
Temperature	–	–	28.5	14.89 ± 0.60 ($n = 11$)
	–	–	29.5	15.25 ± 0.62 ($n = 10$)
	–	–	31.0	9.74[a] ± 0.87 ($n = 3$)

Note: $n_{tot} = 24$ for live embryos; only one observation was obtained for four treatments and one treatment yielded no embryos (calculations for missing cells and modified population means performed using Type IV sums of squares calculations).

[a] Based on modified population marginal mean.

Table 4
Hova-Bator developmental success (%) and hatching success (%) for laboratory incubated eggs ($N = 251$) collected and incubated at different sand moistures and temperatures from Playa Grande and Playa Ventanas, 1997–1998

Moisture (%)	Temperature (°C)	Egg totals	Eggs per incubator				DEV-success (%)	H-success (%)
			DEV	UnDEV	H	UNH		
5	28.5	40	19	21	2	38	47.5	5.0
5	29.5	45	13	32	2	43	28.9	4.4
5	31.0	46	6	40	1	45	13.0	2.2
12	28.5	41	12	29	2	39	29.3	4.9
12	29.5	42	9	33	1	41	21.4	2.4
12	31.0	37	2	35	0	37	5.4	0.0
Totals:		251	61	190	8	243		

Abbreviations: DEV, developed; UnDEV, undeveloped; H, hatched; UNH, unhatched (includes all samples, developed and undeveloped).

ber 16, 1997 were 37.4% and 19.8%, respectively. October 16th corresponded to a period of Spring tides which destroyed nests laid prior to that date. Hova-Bator developmental and hatching success were lower (13.1% and 12.4% lower, respectively) in comparison with the beach (Table 4). Developmental success for artificially incubated eggs differed significantly between treatments. There was a stronger relationship between developmental success and sand temperature ($\chi^2 = 18.34$; $P = < 0.001$) than between developmental success and sand moisture ($\chi^2 = 2.78$; $P = 0.10$). Significant differences in hatching success (7.4%) between treatments (combined effects) could not be accurately determined due to low sample sizes (19 hatchlings out of 251 eggs).

4. Discussion

4.1. Distribution of calcium through incubation

Calcium in *D. coriacea* eggshell decreased by almost 50% during incubation, consistent with similar studies on *C. serpentina* and *Chrysemys picta* (Packard et al., 1984; Packard and Packard, 1986, 1989). Y + A calcium concentration decreased until the last quarter of incubation, but then increased above the initial concentration until hatching. The timing of this increase corresponds to the period during which embryonic metabolic activity and the rate of nutrient mobilization increase prior to hatching (Packard and Packard, 1986, 1987). Transfer of calcium to the embryo, initially withdrawn from the eggshell, is assumed to be responsible for the replenishment of the yolk (Packard and Seymour, 1997). In this study, embryos gained approximately 75% more calcium per gram of body mass than was initially contained within the internal Y + A reserve. However, mean calcium concentrations of hatchlings comprised only 6.5% of the final calcium content of the eggshell (day 60). Calcium loss from the eggshell exceeds that gained by the embryo and is attributed to degeneration of the outer, calcareous layer by internal mechanisms for ionic mobilization (Packard et al., 1984). Calcium moved from the shell into the yolk and albumen of undeveloped eggs, showing that some transport of calcium occurs regardless of the presence of a developing embryo. Why this occurs requires further investigation.

Yolkless leatherback eggs contained approximately one-half of the calcium concentration per gram of eggshell than yolked eggs. Romanoff and Romanoff (1949) reported similar findings for the yolkless eggs of birds. In some cases, the eggshells of yolkless avian eggs have been observed to be either totally or partially undeveloped, owing to a number of factors including low availability of calcium for eggshell formation (Romanoff and Romanoff, 1949; Tuan et al., 1991). Yolkless eggs in *D. coriacea* are often oviposited at the end of egg laying (Rostal et al., 1996; Steyermark et al., 1996), perhaps due to a deficiency in calcium allocation. However, the role and function of yolkless eggs are not well understood.

There was no significant difference in total calcium content of hatchlings from the beach or the laboratory, but the power of the test was low

due to the small sample yielded from the incubators. Therefore, data for hatchlings from the beach and from incubated eggs were combined to increase the overall sample size. Embryos incubated within the laboratory mobilized calcium more slowly during the first half of incubation, but calcium withdrawal increased 12-fold beyond day 30, a trend consistent for all treatments. Packard et al. (1984) reported a similar trend with respect to time for *C. serpentina*, irrespective of the water potential of the substrate.

The total wet mass of fresh eggs was negatively correlated with the amount of calcium in the eggshell, signifying that the amount of calcium allotted to each egg may be constrained. We speculate that the total amount of calcium supplied for eggshell calcification may be portioned equally among the clutch ova. Therefore, smaller eggs may possess thicker shells than larger eggs, with the allotted amount of calcium distributed evenly regardless of size. We further speculate that time-dependent processes may be involved in calcium deposition during passage down the oviduct, which may be illustrated in *Chelonia mydas* with regard to calcification; shell formation begins approximately 72 h following ovulation and is completed within 9–10 days (Miller, 1985).

4.2. Rate of calcium mobilization in relation to treatment

Sand moisture and temperature appear to be important factors that regulate the rate of calcium transport by the CAM. In this study, calcium mobilization was more rapid at low sand moisture and sand temperature than at higher values. Packard and Packard (1986, 1989) demonstrated the opposite with regard to substrate water potential in embryos of C. *picta* and C. *serpentina*, showing calcium withdrawal from the yolk and eggshell was faster in wetter environments over a comparable incubation period. *Dermochelys coriacea* nests predominately during the dry season on both coasts of Costa Rica, and thus the mechanisms controlling ossification in the embryo may have evolved at lower sand moistures, as well as favoring lower sand temperatures closest to the pivotal sex determining temperature. The combined effects of moisture and temperature ($m \times t$), however, were statistically insignificant. This suggests that either calcium mobilization is influenced more by a single factor, or that the samples analyzed were too few in number.

The effect of incubation temperature and substrate water potential on ionic mobilization within the egg in this study did not concur with those previously reported in chicken, *Gallus gallus* (Tuan et al., 1991), and snapping turtle (*C. serpentina*) eggs (Packard and Packard, 1989). Possible hormonal responses sensitive to hydration require investigation, as well as related factors that may be present within leatherback eggs (or other sea turtle eggs) not occurring in freshwater turtles.

4.3. Developmental success and hatching success

Reasons for the low developmental success and hatching success are unclear. However, developmental success was difficult to assess by visual examination, especially in natural nests. The three-stage field technique typically underestimates true developmental success (B. Bell, personal communication). Degeneration of egg contents and potential embryos may have been responsible for the developmental success rates of 37.4% on Playa Grande and 24.1% for the laboratory. Lower laboratory developmental success may also be due to handling of the egg delaying development slightly. Reasons for developmental failure are often difficult to determine, but include both intrinsic and extrinsic factors (Wyneken et al., 1988).

Hatching success for leatherback nests at Playa Grande and other locations has typically been in the range of 50% or greater, with discernable embryos (live and dead) approaching 70% or more (Leslie et al., 1996; Binckley et al., 1998). Flooding of nests was almost certainly a major cause of embryonic mortality in the early part of the 1997/1998 nesting season. Developed eggs and hatchlings were observed only from clutches oviposited after the mid- to late-October tides, which destroyed the entire assemblage of nests at Las Baulas, probably due to increased chlorinity (Bustard and Greenham, 1968). Hatching success is also affected by the local conditions of the beach, including sand moisture content, sand-grain size, sand organic content and biotic contamination (Leslie et al., 1996). The nesting environment of Playa Grande and Playa Ventanas during the 1997–1998 nesting season experienced continual changes in beach dynamics, as opposed to more stable condition of high sand deposition

and greater availability of nesting areas in previous seasons (personal observation). Factors such as the shifting beach sand, removal of extensive open beach areas, partial or total covering of nesting areas with debris associated with high tides, and thick vegetation cover may have contributed to low developmental and hatching success. These conditions are not generally encountered in most nesting years.

Results of egg incubation showed that sand temperature had a significant influence upon the successful development of *D. coriacea* embryos. Low sand temperatures were associated with increased incubation period and slower growth rates (28.5°C > 29.5°C > 31.0°C). Substrate temperature and water potential directly regulate embryonic development of *C. picta* and *C. serpentina* (Morris et al., 1983; Packard et al., 1985), but wetter conditions and warmer temperatures produced greater success. These studies have also shown that eggs of both *C. picta* and *C. serpentina*, when exposed to wet substrates (−130 kPa water potential), have higher hatching success and longer incubation periods than eggs incubated in drier substrates (≥ −650 kPa). In addition, increased substrate temperature decreases the incubation period for these species and increases the rate of nutrient uptake (Packard and Packard, 1986, 1987, 1989). In our study, we used sand as the nesting substrate, which is more sensitive to fluctuations in moisture content. Sand moisture tensions recorded for Playa Grande during the nesting season are much higher than the tensions in the above studies (7.5% H_2O or −12 kPa in mid-October and 5.5% H_2O or −25 kPa in late January; measured at average nesting depth = 68.9 cm). We speculate that *D. coriacea* has evolved a developmental strategy to exploit the decrease in water potential observed during their nesting season.

The nesting of Pacific leatherbacks on both coasts of Costa Rica during drier seasons may be indicative of unique physiological adaptations to the warm ocean-beach environment and species-specific requirements. This physiological adaptation to dry season nesting is true for all species of marine turtles, suggesting that embryos of marine turtles may have evolved the ability to resist water loss during dry incubation periods or in dry, sandy environments. The majority of Pacific leatherback nesting occurs during the dry season months of November through February, suggesting that the environmental conditions required for development may be more favorable than for wetter periods. This, we speculate, may have had an evolutionary impact on this species and may limit its nesting to tropical circumglobal regions experiencing dry periods. Freshwater turtles, often localized to wetland habitats, may be adapted to wetter conditions and benefit from substrates containing a higher moisture content. However, in both marine and freshwater turtles, the conditions most favorable for optimal evaporative water loss and gas exchange must be present in order to ensure successful development and hatching (Ackerman, 1997).

Our results show that the nest environment has significant influence on the mobilization of calcium and developmental success of leatherback eggs. Calcium mobilization during development is critical for embryonic ossification and may be impeded by adverse environmental conditions. In turn, mobilization of ionic nutrients and gas exchange in nests possessing optimal physical conditions can influence the degree of developmental success for the leatherback embryo. This stresses the importance of properly selected and monitored environmental conditions in artificial hatcheries employed as a conservation measure, as well as the utility of nest relocation operations on beaches with regions subject to periodic tidal inundation, in order to maximize effectiveness.

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