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Calcium Depletion of Eggshell After Fungal Invasion of Sea Turtle Eggs

ANDREA D. PHILLOTT¹, C. JOHN PARMENTER¹,
AND STEPHEN C. MCKILLUP¹

¹*School of Biological and Environmental Sciences, Central Queensland University, Rockhampton, Queensland 4702 Australia
[a.phillott@cqu.edu.au, j.parmenter@cqu.edu.au,
s.mckillup@cqu.edu.au]*

ABSTRACT. – The calcium content of flatback (*Natator depressus*), loggerhead (*Caretta caretta*), hawksbill (*Eretmochelys imbricata*), and green (*Chelonia mydas*) sea turtle eggshells are not significantly different. Green turtle eggs with external fungal invasion by *Fusarium solani* had significantly reduced calcium content of the outer, inorganic portion of the eggshell.

The developmental failure of sea turtle eggs has often been related to fungal invasion (Solomon and Baird 1980; Wyneken et al. 1988; Eckert and Eckert 1990; Mo et al. 1990; Acuña-Mesén 1992; Peters et al. 1994; Phillott and Parmenter 2001), but the exact cause of embryo mortality is still unknown. Solomon and Baird (1980) suggested hyphal penetration of the eggshell and eggshell membranes may impair gaseous exchange, invade embryonic tissue, and/or impede normal embryonic development by depleting the amount of calcium in the eggshell. Phillott and Parmenter (2001) concluded that if hyphal impediment of gas exchange occurs, the severity of its influence would depend upon the size and location of the fungal growth, the sea turtle species and egg size. Fungi are capable of penetrating the eggshell and invading embryonic tissue (Phillott 2004). The potential for eggshell calcium depletion by fungi and the subsequent influence on embryogenesis have not been quantitatively established.

Solomon and Baird (1980) observed fungal hyphae between the soft shell membrane and crystalline shell layer in green sea turtle eggs. They concluded that the high calcium content of these hyphae, in conjunction with their proximity to the calcified eggshell, suggested fungi may be extracting calcium from the eggshell, thereby causing a deficiency in the embryo and impairing normal development.

Early in the growth phase during the second half of incubation, turtle embryos initially obtain calcium from the egg yolk. The yolk is quickly depleted of calcium, which must then be mobilized from the eggshell during the last trimester (Packard 1994; Sahoo et al. 1998). Calcium is the major inorganic constituent of sea turtle eggshell (20%–21%) (Solomon and Baird 1976; Sahoo et al. 1998), and the majority required for embryogenesis is derived from this source (60%—Sahoo et al. 1998; 62%—Bustard et al. 1969; 75%—Simkiss 1962). Consequently, calcium depletion could be expected to influence not only embryonic development (Solomon and Baird 1980) but also eggshell structure (Sahoo et al. 1996).

Sea turtle eggshell consists of 2 layers: an outer inorganic surface composed of organized, crystalline aggregates of calcium carbonate in the aragonite form (Solomon and Baird 1976) and an underlying organic component composed of the shell membrane or *membrana testacea*, consisting of dense fibrous organic matter (Packard and Packard 1979; Sahoo et al. 1996) with a

Table 1. Calcium content of hawksbill, loggerhead, flatback, and green sea turtle eggshell ($n=5$ for each species).

Species	Calcium content of eggshell (% by weight)			
	Organic component		Inorganic component	
	Mean	SD	Mean	SD
Hawksbill	9.02	9.57	43.06	10.94
Loggerhead	9.78	7.33	39.56	7.98
Flatback	9.01	9.73	43.37	8.04
Green	4.66	4.23	40.09	7.03

thin basement membrane (Sahoo et al. 1996). There is no cuticle on the egg exterior, and no distinct pore structure (Solomon and Baird 1976). The eggshell is the egg's first defense against microbial invasion, and, as such, its integrity is of great importance.

To determine the potential for fungal depletion of calcium from sea turtle eggshell, the calcium concentration of eggshell from oviposited eggs was compared with that of eggs that had been colonized by fungi but had not lost any of their calcium to the developing embryo.

Methods. — To estimate the calcium concentration of sea turtle eggshells, 1 egg was collected directly from a nesting female from each of 5 flatbacks (*Natator depressus*, Peak Island, 23°20.5'S, 150°56'E), loggerheads (*Caretta caretta*, Mon Repos Conservation Park, 24°48'S, 152°27'E), hawksbills (*Eretmochelys imbricata*, Milman Island, 11°10'S, 143°00'E), and greens (*Chelonia mydas*, Heron Island, 23°26'S, 152°27'E). Eggs were immediately frozen to -5°C for storage. The sample size was limited by permit regulations on the number of viable eggs allowed to be collected.

Prior to analysis, eggs were thawed to room temperature and their contents removed. The eggshells were air-dried and mounted on double-sided carbon tape attached to an aluminium stub and examined with a Jeol JSM-5300LV Scanning Microscope to determine calcium content. Energy dispersive X-ray analysis was conducted at an accelerating voltage of 15kV, with acquisition on an area of 10 × 10 μm at ×1000 magnification, for 60 sec. Five fragments from each shell were analyzed to determine the percentage concentration of calcium. Statistical analysis of eggshell calcium content among species was carried out using a one-way nested ANOVA (with eggs as a nested factor within species).

To determine the calcium content of eggshells affected by fungus, 5 eggs from a single clutch of *C. mydas* (that had been collected for other research purposes) were analyzed for shell calcium. The clutch was collected at Heron Island and stored at 8°C for < 48 hrs at the Heron Island Research Centre before transport to Central Queensland University, following the procedures of low-temperature transport described by Harry and Limpus (1989). None of the eggs developed a white spot under incubation conditions of 28°C upon a natural sand

Table 2. Calcium content of nonaffected and fungal-affected green sea turtle eggshell ($n=5$ for each category).

Fungal Presence	Calcium content of eggshell (% by weight)			
	Organic component		Inorganic component	
	Mean	SD	Mean	SD
Yes	4.84	4.46	33.36	5.97
No	4.66	4.23	40.09	7.03

substrate, indicating failure of embryonic development beyond the gastrula present at oviposition (Decker 1967; Mahmoud et al. 1973; Ewert 1985). Fungus appeared on a single egg 7 days after being placed in the incubator and spread through the entire egg mass during the following 4 weeks. At this time 5 eggs with fungus visible on their exterior were randomly selected and 5 fragments per egg analyzed as described previously. Statistical analyses of eggshell calcium content among eggs with and without fungi was conducted using a one-way nested ANOVA (with eggs as a nested factor within fungal presence/absence) following arc-sine transformation to normalize these percentage data. Fungal hyphae were scraped from 5 different locations of each egg exterior with a sterile scalpel blade and mounted on double-sided carbon tape for calcium analysis. The fungus was identified following Booth (1971).

Results. — The calcium contents of unaffected eggs from each species are given in Table 1. There was no significant difference in calcium concentration of the eggshell inorganic or organic component among species (inorganic $F_{3,16} = 1.180$, $p > 0.05$; organic: $F_{3,16} = 1.384$, $p > 0.05$) or among the eggs nested within each species (inorganic component $F_{16,80} = 1.091$, $p > 0.05$; organic component $F_{16,80} = 1.692$, $p > 0.05$).

Fungal growth (by *Fusarium solani*) on the exterior of green sea turtle eggs (see Table 2) significantly reduced the calcium content of the outer, inorganic layer, but did not alter that of the inner, organic membrane (nested ANOVA: inorganic component $F_{1,8} = 11.223$, $p = 0.010$; organic component $F_{1,8} = 0.016$, $p > 0.05$). There was no difference among eggs nested within the two categories (inorganic component $F_{8,40} = 1.183$, $p > 0.05$; organic component $F_{8,40} = 1.263$, $p > 0.05$). SEM imagery of the fragments during calcium analysis indicated colonization by *F. solani* was superficial and did not penetrate the calcified layer into the shell membrane. The calcium concentrations of 5 samples of *F. solani* hyphae removed from the eggs are given in Table 3.

Pure calcium carbonate (Fisons Analytical Reagent, Code C/1120/53, Batch 9335052) was used to verify the precision of the Jeol microprobe calcium analysis. Calcium content was determined as 42.11% ± 1.96% (mean ± SD, $n = 5$) as compared to 40.08% actual.

Table 3. Calcium concentration of the fungus *F. solani* removed from the exterior of green sea turtle eggs.

Egg No.	Calcium content (% by weight)		n
	Average	SD	
1	2.16	0.48	5
2	3.32	0.83	5
3	5.56	3.53	5
4	2.18	0.55	5
5	2.76	0.91	5
Average	3.20	2.01	

Discussion. — This is the first report of calcium analyses for *E. imbricata*, *C. caretta*, and *N. depressus* sea turtle eggs. Solomon and Baird (1976) reported calcium to be 20% of *C. mydas* eggshell, but their analysis was performed on homogenized eggshell that included both the organic and inorganic components. Calculating the results of the present study in the same fashion gives an average of 19.15% calcium for *C. mydas* eggshell, which is almost identical to the results of Solomon and Baird (1976).

Although high variability in calcium content was detected in the organic and inorganic components of eggshell from all species (Table 1), there was no visual evidence of amorphous calcium deposits attributed to "calcium splash" (Chan and Solomon 1989). This eggshell phenomenon occurs in birds after delayed oviposition (often due to stress), resulting in irregularly distributed superficial and superfluous calcium. In sea turtles, delayed oviposition may occur after disturbance during nesting (by humans or other turtles), or through failure of nest construction due to unsuitable nesting substrate. However, this is unlikely to result in calcium splash because shell formation is already complete and the eggs are distal to the shelling region of the oviduct by this time. Calcium splash in sea turtles could only occur prior to nesting emergence, when severe disturbance may result in the cessation of oviductal motility during shelling and cause extra calcium to be deposited on the shell membrane or eggshell exterior. This would result in elevated calcium levels on isolated areas of a single egg.

Fusarium solani is 1 of 3 species of fungi regularly identified from failed eggs of turtles nesting in eastern Australia (Phillott et al. 2001, 2004) and has been implicated in embryo mortality (Phillott and Parmenter 2002). Its source was probably the natural sand substrate upon which the eggs were incubated (see Phillott and Parmenter 2002). Since there was no post-oviposition embryonic development, the calcium loss demonstrated by eggs affected by fungus can only be attributed to fungal presence, although ideally it would have been useful to compare calcium contents of eggs that had failed to develop but had not been subsequently colonized by fungi.

The similarity of eggshell calcium levels (this study) and structure (A.D. Phillott, unpub. data) of the 4 turtle species investigated suggests fungal depletion of calcium

is likely to be similar across all cheloniid species of sea turtle. Because the fungus was superficial on the egg and did not reach the shell membrane, it is not surprising that there was no calcium depletion of the latter. Analysis after a longer period of fungal presence would possibly show decreased levels of shell membrane calcium if the eggshell were eventually penetrated by fungus.

Two other species of fungi (*Fusarium oxysporum* and *Pseudallescheria boydii*) are also commonly isolated from failed sea turtle eggs in eastern Australia (Phillott et al. 2001, 2004). Their ability to cause calcium depletion of sea turtle eggshell requires further investigation.

Calcium requirements for fungal growth are poorly known. Available evidence is contradictory (Harold 1994) and regarded as both a macro- (Garraway and Evans 1984) and micro- (Jennings and Lysek 1996) nutrient. Uptake from the external medium may occur by facilitated diffusion, proton symport, or pinocytosis across the absorption zone of the hyphal tip (reviewed by Garraway and Evans 1984).

The effect of depleted eggshell calcium on the developing turtle embryo is poorly understood. Because the embryo does not extract calcium from the eggshell until the third trimester, it is assumed that embryonic development would proceed normally (with respect to calcium dynamics) until then, in the absence of other pathological factors. However, once calcium demand increases for osteogenesis, its insufficiency could possibly result in teratogenesis and/or embryo mortality.

Eggshell exfoliation of viable eggs is normally observed in the week prior to hatching (Miller 1982) and occurs due to calcium mobilization from the eggshell by the rapidly maturing embryo (Simkiss 1962). On inspection after hatching, the calcified layer of the eggshell appears disrupted (Schleich and Kästle 1988; Sahoo et al. 1996) as a result of calcium depletion (Sahoo et al. 1996). Premature loss of eggshell integrity, due to fungal depletion of calcium, would weaken its function as a barrier and allow easier hyphal penetration and subsequent access to the nutrient-rich yolk and embryonic material. Ultimately, both depletion of available calcium and/or destruction of the eggshell's integrity are likely to result in embryo mortality.

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