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FUNGAL COLONIZATION OF GREEN SEA TURTLE (*CHELONIA MYDAS*) NESTS IS UNLIKELY TO AFFECT HATCHLING CONDITION

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***Abstract.*—To determine the possible effect of fungal invasion on Green Sea Turtle (*Chelonia mydas*) embryo development and subsequent hatchling condition, we compared hatchling scalation patterns, straight carapace length, and weight among nests with varying proportions of fungal colonization. Analyses suggested that the condition of hatchlings emerging from nests that have a high percentage of eggs colonized by fungus was similar to those from nests without fungus.**

Key Words.—*Chelonia mydas*; egg; embryo; fungal invasion; Green Sea Turtle

INTRODUCTION

Three species of fungi, *Fusarium oxysporum*, *F. solani*, and *Pseudallescheria boydii*, have been isolated from failed sea turtle eggs in eastern Australia (Phillott et al. 2001, 2004). Fungal invasion of sea turtle nests followed mortality of an egg from other causes. Using this failed egg as a nutrient focus, hyphae were able to spread to adjacent eggs and potentially encompass the entire egg mass (Phillott and Parmenter 2001a). The process by which fungi directly caused embryo mortality has not been determined; possibilities include hyphal penetration of the eggshell and eggshell membranes (Phillott 2004) thus impairing gaseous exchange (Phillott and Parmenter 2001b), invasion of embryonic tissue (Solomon and Baird 1980; Phillott 2004), and/or impeding normal embryonic development by depleting the amount of calcium in the eggshell (Phillott et al. 2006). The close proximity of eggs within the sea turtle nest could also allow fungal growth to influence eggs without direct contact. For example, volatile mycotoxins or other metabolites, that originated from fungal growth on the exterior of one egg, could have affected adjacent eggs, and had a detrimental effect on the development and condition of hatchlings.

Hatchling condition or fitness has often been estimated from hatchling size or weight (Shine 2004). However, since scute abnormalities may reflect genetic or teratogenic factors (reviewed by Velo-Antón et al. 2011), patterns of scalation on the shell (carapace and plastron) and head are also used (Davis and Grosse 2008). In the absence of other lethal abnormalities, scute abnormalities are unlikely to cause mortality (Miller 1982) but a higher frequency of anomalous scales occurs in unhatched sea turtle embryos compared to hatchlings, which have a higher frequency of scale anomalies than adults (Andrea Phillott, unpubl. data). The presence of anomalous scales may, therefore, indicate physical or physiological defects that affected fitness and reflect mutations or developmental abnormalities due to the presence of fungus.

In this study we compared hatchling morphology among nests with varying degrees of fungal colonization to indirectly assess the potential influence of fungal invasion on hatchling fitness. A change in the most common species of nest fungi from *F. solani* in the 1996–97 nesting season to *P. boydii* in the 1997–98 nesting season at our study site (Phillott et al. 2004) also allowed us to compare whether *F. solani* and *P. boydii* might have had different sub-lethal effects on the development of turtle embryos. The study was conducted under natural conditions in the field to determine if more controlled laboratory investigations, requiring the collection and sacrifice of eggs, would be warranted.

MATERIALS AND METHODS

Hatchlings were selected at random from Green Sea Turtle clutches during emergence, or while they were crossing the nesting beach at Heron Island, Australia (23° 26' S, 151° 55' E). After capture, we immediately weighed hatchlings (to 0.01 g) on an electronic balance and we measured their straight carapace lengths (SCL) with Vernier calipers (to 0.1 cm). We counted and recorded the number of nuchal, vertebral, postvertebral, costal, marginal, gular and inframarginal scales of the carapace and postocular, preocular, prefrontal and postparietal scales of the head. We then released the hatchlings at their point of capture.

We detected emerged nests by the presence of characteristic sinkholes that formed with the reduction of nest volume at hatchling emergence, by the presence of hatchling tracks, or by visually observing hatchlings emerging from the nest. We removed the

TABLE 1. Green Turtle (***Chelonia mydas***) hatchling straight carapace length (SCL) and weight in the 1996–97 and 1997–98 nesting seasons at Heron Island, Australia (Mean ± SD). Hatchling SCL (t = -6.521, df = 597, $P < 0.001$) and weight (t = -6.371, df = 597, $P < 0.001$) were significantly lower in the 1996–97 season than in 1997–98.

	Nesting Season	
	1996–97 (n = 287)	**1997–98 (n = 312)**
SCL (cm)	4.8 ± 0.2	4.9 ± 0.2
Weight (g)	24.07 ± 2.28	25.43 ± 2.88

contents of the egg chamber by hand and sorted it into eggshell (greater than half the size of an egg at oviposition), unhatched eggs (entire eggs with egg contents that may or may not contain visible signs of embryonic development), and depredated eggs (entire eggs without their egg contents, and a small perforation often the only indication of depredation). We recorded counts of all eggs/eggshells meeting these criteria for each nest and used them to calculate the following:

Clutch count = Eggshell + Unhatched Eggs + Depredated Eggs

Hatch Success = (Eggshell / Clutch Count) × 100

We kept separate counts of unhatched eggs invaded by fungi, as apparent by a black growth visible macroscopically on the egg exterior. This allowed the calculation of:

% Failed Eggs with Fungi =
(Unhatched eggs with fungi / Total unhatched eggs) × 100

% Clutch with Fungi =
(Unhatched eggs with fungi / Clutch count) × 100

We measured nest depth, from the beach surface to the bottom of the excavated egg chamber using a flexible tape measure.

We used independent sample t-tests to compare hatchling SCL and weight between the two nesting seasons. We analysed each season's scale count data separately because the prevalent fungi changed between seasons (Phillott et al. 2004). We compared the number of scales with the normal scale pattern of Green Turtles (Marquez 1990) and we determined the number of sub- and super-numerary scales for each scale type. We then assigned each hatchling to one of the following categories: 0, 1, 2, 3, 4, 5, 6, or 7 anomalous scales. For example, we assigned a hatchling with no anomalous scales was to the category 0; a hatchling with anomalous counts in 2 scale types (e.g. an extra nuchal and one less vertebral) to the category of 2; a hatchling with anomalous counts in 3 scale types (e.g., an extra nuchal, one less vertebral and an extra marginal) to the category of 3 etc. etc..

TABLE 2. The number of Green Turtle (*Chelonia mydas*) hatchlings with anomalous scale counts at Heron Island, Australia in the 1996–97 and 1997–98 nesting seasons.

	# Hatchlings	
# of Scale Categories with Anomalies	**1996–97 Season (n = 287)**	**1997–98 Season (n = 312)**
0	73	160
1	106	83
2	79	49
3	20	13
4	6	6
5	2	1
6	0	0
7	1	0

We used Chi-square analysis to compare the numbers of hatchlings in these categories between the two seasons. We omitted categories containing zero counts in both seasons from the analysis since they are unsuitable for inclusion (Zar 1999). We used a 2 × 2 Chi-square with Yates correction for continuity to compare the numbers of hatchlings with and without scale abnormalities between the 2 seasons. We used Spearman's rank correlation to determine whether the number of anomalous scale categories varied significantly with hatchling straight carapace length, hatchling weight, nest hatch success, the percentage of failed eggs with fungi and percentage of the clutch with fungi. We set α at $P < 0.05$.

RESULTS

We collected a total of 287 hatchlings (8–10 per clutch) from 29 Green Sea Turtle (*Chelonia mydas*) clutches in the 1996−97 nesting season, and 312 hatchlings from 32 clutches in the 1997–98 nesting season. Hatchling SCL (t = -6.521, df = 597, $P < 0.001$) and weight (t = -6.371, df = 597, $P < 0.001$) were significantly lower in the 1996–97 nesting season than in 1997–98 (Table 1). The distribution of hatchlings in the anomalous scale count categories (Table 2) also differed significantly between the two nesting seasons (χ^2 = 44.17, df = 6, $P < 0.0001$). We present a more detailed account of the occurrence of anomalies in Tables 3 and 4. The percentage of hatchlings with scale anomalies was 75% in 1996–97 but decreased significantly (χ^2 = 42.04, df = 1, $P < 0.0001$) to 49% in 1997–98. The outstanding changes in category values were the occurrence of sub-numerary postoculars (-1 both left and right) and asymmetry of the postparietal in the first season.

There were no significant correlations between the number of anomalous scale categories and any hatchling or nest characteristic in the 1996–97 season. The only significant correlation that occurred in the 1997–98 nesting season was with the percentage of

TABLE 3. Scale categories of Green Turtle (*Chelonia mydas*) hatchlings showing anomalous counts in the 1996–97 nesting season at Heron Island, Australia.

Scale Category		Normal Count	% of Hatchlings with Sub- and Super-numerary Scales					
			-2	-1	0 (normal)	+1	+2	+3
Nuchal		1	-	0	100	0	0	0
Vertebral		5	0	1	98	1	0	0
Postvertebral		2	0	0	100	0	0	0
Costal	left	4	0	0	97	2	0	0
	right	4	0	0	97	3	0	0
Marginal	left	11	0	1	99	0	0	0
	right	11	0	0	99	0	0	0
Postocular	left	4	0	10	80	10	0	0
	right	4	0	10	77	12	0	0
Preocular	left	0	-	-	100	0	0	0
	right	0	-	-	100	0	0	0
Prefrontal		2	0	0	100	0	0	0
Postparietal		2	0	2	84	11	2	0
(symmetry)[a]		S	-	-	44	56	-	-
Inframarginal	left	4	0	0	98	2	0	0
	right	4	0	0	100	0	0	0
Gular		1	-	0	100	0	0	0

- values in this group are not possible (e.g. a normal scale count of "0" cannot have variation that is less than this)
[a]0 = scale(s) symmetrical; +1 = scale(s) asymmetrical
Note: rounding may result in category totals not equalling 100%

failed eggs with fungi (Table 5).

DISCUSSION

Hatchling SCL and weight differed significantly between the two nesting seasons but there was no significant correlation between these variables and the occurrence of anomalous scales. Variation in hatchling size and weight between seasons was probably due to differing thermal and hydric conditions during incubation as a result of rainfall (1996–97: 237 mm, 1997–98: 183 mm) and ambient temperature (±SD 1996–97: max. 29.8 ±1.7° C, min. 23.1 ±1.6° C, 1997–98: max. 30.9 ±2.0° C, min. 24.0 ±1.9° C; Phillott et al. 2004). Such differences in thermal and hydric micro-climatic conditions were also one possible cause of the occurrence of scale anomalies, which decreased significantly from 1996–97 to 1997–98. Although the mechanisms have not been determined, experimental studies have shown that carapacial abnormalities can occur be attributed to environmental conditions (relatively higher nest temperatures and/or lower substrate moisture levels) during incubation, pollutants, and/or loss of genetic diversity in bottlenecked populations (reviewed in Velo-Antón et al. 2011). Synergistic or antagonistic interactions between thermal and hydric nest conditions, hatchling weight and SCL, and carapacial anomalies have yet to be explored, but results from this study suggest they may be complex, even in the absence of additional factors such as fungal invasion of the nest.

TABLE 4. Scale categories of Green Turtle (***Chelonia mydas***) hatchlings showing anomalous counts in the 1997–98 nesting season at Heron Island, Australia.

Scale Category		Normal Count	% of Hatchlings with Sub- and Super-numerary Scales					
			-2	-1	0 (normal)	+1	+2	+3
Nuchal		1	-	0	99	1	0	0
Vertebral		5	0	0	98	2	0	0
Postvertebral		2	0	0	100	0	0	0
Costal	left	4	0	0	99	1	0	0
	Right	4	0	0	99	1	0	0
Marginal	left	11	0	1	100	0	0	0
	Right	11	0	0	100	0	0	0
Postocular	left	4	0	5	83	13	0	0
	Right	4	0	6	80	14	0	0
Preocular	left	0	-	-	100	0	0	0
	Right	0	-	-	100	0	0	0
Prefrontal		2	0	0	100	0	0	0
Postparietal		2	0	2	87	11	2	0
(symmetry)[a]		0	-	-	80	20	-	-
Inframarginal	left	4	0	1	98	1	0	0
	right	4	0	0	100	0	0	0
Gular		1	-	0	100	0	0	0

- values in this group are not possible (e.g. a normal scale count of "0" cannot have variation that is less than this)
[a]0 = scale(s) symmetrical; +1 = scale(s) asymmetrical
Note: rounding may result in category totals not equalling 100%

There are two possible reasons for the single significant Spearman rank correlation in Table 5. Firstly, the significant correlation may be real, and the number of anomalous scale categories in the 1997–98 nesting season was influenced by the percentage of failed eggs colonized by *P. boydii*. Secondly, since the analyses in Table 5 are of 10 separate and independent Spearman rank correlations, under an $\alpha = 0.05$ there is a 63% probability of a Type I error (Zar 1999). Therefore, we believe the significant correlation in the 1997–98 season to be a result of the presence of *P. boydii* on failed eggs within the nest or a Type I error. The single significant correlation does not allow a strong comparison of the effects of *F. solani* and *P. boydii* colonization of sea turtle eggs on the development of turtle embryos, and although we tentatively conclude that any effect of either fungus on abnormal scale counts was weak or non-existent, further work would be required to reach a more certain conclusion.

The species of fungus dominant on failed sea turtle eggs in each season (1996–1997: *F. solani*; 1997–1998: *P. boydii*) may have been influenced by differences in thermal and hydric conditions or fungal competition (Phillott et al. 2004); *F. solani* is not usually prevalent in regions with relatively high rainfall and low temperatures (Burgess and Summerell 1992), as experienced in the 1996–97 nesting season, but is out-competed by *P. boydii* when artificially incubated within thermal and hydric nest conditions. Differing environmental conditions, and/or fungal dominance, may interact synergistically to influence carapacial abnormalities in sea turtle hatchlings; further research in this field would require laboratory studies to control for the different combinations of variables.

Patino-Martinez et al. (2012) concluded that exposure of sea turtle eggs to fungus in the first and middle trimesters of incubation resulted in smaller hatchlings but did not lower hatch success. However, their exposure technique of applying fragments (size and number not described) of contaminated eggshell to the exterior of viable eggs, may have reduced the available respiratory surface area and directly reduced embryonic development (see Phillott and Parmenter 2001b); a control application of uncontaminated eggshell to the egg exterior was not included in the study. The role of fungus in the reduced hatch success observed by Patino-Martinez et al. (2012) is therefore difficult to understand. Building upon the results from Patino-Martinez et al. (2012) and this study, we suggest future research carefully consider the method of egg exposure to potential pathogens, and include controlled conditions that would determine possible interactions between incubation environment and fungal colonisation of the nest, and subsequent effects on hatchling weight, SCL and/or condition.

In summary, the current study suggests the effects of fungal invasion of sea turtle nest may be localized to colonized eggs only. The number of anomalous scales possessed by a hatchling does not appear to be related to nest hatch success and evidence for an effect of fungus was very weak. Since hatchlings emerging from nests that have a high percentage of failed eggs colonised by fungi did not show a significant increase in abnormal scalation, or variation in SCL or weight, they should have a similar fitness to those from nests without fungi.

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TABLE 5. Spearman rank correlation-coefficients between the number of anomalous scale categories and hatchling/nest characteristics for Green Turtles (*Chelonia mydas*) in the 1996–97 and 1997–98 nesting seasons at Heron Island, Australia. Statistically significant results are in bold.

	Nesting Season	
	1996–97 (n = 287)	**1997–98 (n = 312)**
Hatchling SCL	$r_s = -0.011$, $P = 0.850$	$r_s = 0.057$, $P = 0.316$
Hatchling Weight	$r_s = 0.026$, $P = 0.660$	$r_s = 0.078$, $P = 0.167$
Hatch Success	$r_s = 0.057$, $P = 0.338$	$r_s = 0.059$, $P = 0.298$
% Failed Eggs with Fungi	$r_s = 0.027$, $P = 0.650$	**$r_s = 0.126$, $P = 0.026$**
% Clutch with Fungi	$r_s = 0.014$, $P = 0.815$	$r_s = -0.031$, $P = 0.583$

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