FUNGAL COLONISATION OF SEA TURTLE NESTS IN EASTERN AUSTRALIA

by

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ABSTRACT

This thesis reports on a study of fungi found in nests of green (*Chelonia mydas*), loggerhead (*Caretta caretta*), flatback (*Natator depressus*) and hawksbill (*Eretmochelys imbricata*) sea turtle nests at Heron Is., Wreck Is., Mon Repos, Peak Is. and Milman Is., eastern Australia, examined during the 1995/96-1998/99 nesting seasons. Egg mortality and fungal colonisation of eggs were significantly greater in loggerhead turtle nests at Heron Is. and Wreck Is. than in green turtle nests at the same two rookeries, and also greater than in loggerhead, green, flatback and hawksbill turtle nests at other rookeries.

Three species of fungi, *Fusarium oxysporum*, *Fusarium solani* and *Pseudallescheria boydii* were frequently isolated from failed eggs in nests of all turtle species at all rookeries. The fungi are all ubiquitous soil species, and probably originated from the nest substrate. However, there was some evidence for acute, intra-seasonal oviductal contamination of eggs with fungi, accumulated in the cloaca and oviduct during nesting behaviour. The *Pisonia grandis* forest and high seabird density, present only at Heron Is. and Wreck Is. of all the rookeries investigated, did not appear to harbour fungi colonising sea turtle eggs at these locations.

Within the sea turtle nest, fungi first appeared on an egg that had failed from other (natural) causes. Spores and hyphal fragments in the sand are likely to be disturbed during the nesting process and may settle on the exterior of sea turtle eggs. Experiments showed that the infection of viable eggs of all sea turtle species with fungi is possibly inhibited by the anti-fungal properties of mucus secreted during oviposition, egg albumen and the dense ultra-structure of the eggshell. However, if

an egg dies the lytic enzymes and acids produced by *F. oxysporum*, *F. solani* and *P. boydii* could allow penetration of the eggshell and utilisation of egg contents. Using this nutrient source, hyphae could then expand to adjacent, viable eggs. The linear growth rate of all fungi varied with the thermal and hydric conditions during incubation.

Embryo mortality as hyphae spread across a viable egg is probably due to inhibition of the respiratory surface area or possibly to calcium deprivation. Analyses of hatchlings from nests with different levels of fungal colonisation suggested that hatchlings emerging from nests that have a high percentage of eggs colonised by fungi should have a similar fitness to those from nests without fungi.

Any nest characteristic that enhances egg failure, particularly early in incubation, will markedly accelerate fungal invasion of the sea turtle nest. Each additional failed egg greatly increases the likelihood of a focus of growth being available for fungi.

The increased vulnerability of loggerhead nests at Heron Is. and Wreck Is. to egg failure and subsequent colonisation by fungi is likely to be to due characteristics of the nest. Significantly high substrate conductivity at Heron Is. and Wreck Is. is likely to impose osmotic stress on loggerhead eggs, which are smaller than green turtle eggs in adjacent nests, and result in higher egg mortality. Once fungal invasion of the nest is established, equivalent linear growth of fungus will cover a greater surface of the egg and allow faster access to adjacent eggs in loggerhead turtle nests than green turtle nests. This results in a significantly lower hatch success of loggerhead turtle nests.

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DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made in the text.

Signature Redacted

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A.D. Phillott

PUBLICATIONS

Publications resulting from this study are as follows:

Chapter 4

Phillott A.D., Parmenter C.J. and Limpus C.J. 2001. Mycoflora identified from failed green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtle eggs at Heron Island, Australia. Chel.Cons.Biol. 4: 170-172.

Phillott A.D., Parmenter C.J. and Limpus C.J. 2002. The occurrence of mycobiota in eastern Australian sea turtle nests. Mem.Qd.Mus. In Press.

Chapter 5

Phillott A.D. 2001. *Pisonia grandis* does not appear to harbour fungi known to invade sea turtle nests at Heron Island, eastern Australia. Trans.R.Soc.S.A. 125: 69-70.

Phillott A.D. and Elsmore S.A.M. Submitted. Black noddies (*Anous minutus*) and wedge-tailed shearwaters (*Puffinus pacificus*) as potential hosts for fungi invading sea turtle nests at Heron Island, Queensland. Trans.R.Soc.S.A.

Phillott A.D., Parmenter C.J., Limpus C.J. and Harrower K.M. 2002. Mycobiota as Acute and Chronic Cloacal Contaminants of Female Sea Turtles. Aust.J.Zool. 50: 687-695.

Chapter 6

Phillott A.D. and Parmenter C.J. 2001. The distribution of failed eggs and appearance of fungi in artificial nests of green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) sea turtles. Aust.J.Zool. 49: 713-718.

Phillott A.D. and Parmenter C.J. Submitted. Deterioration of green sea turtle (*Chelonia mydas*) eggs after known embryo mortality. Chel.Cons.Biol.

Chapter 7

Phillott A.D. and Parmenter C.J. 2001. Influence of diminished respiratory surface area on survival of sea turtle embryos. J.Exp.Zool. 289: 317-321.

Phillott A.D., Parmenter C.J. and McKillup S.C. Submitted. The potential for calcium depletion of eggshell after fungal invasion of sea turtle eggs. Chel.Cons.Biol.

Appendix C

Phillott A.D. 2002. Minimizing fungal invasion during the artificial incubation of sea turtle eggs. Herp.Rev. 33: 41-42.

FOREWORD

The arrangement of this thesis is unusual in that it is composed of many, small chapters rather than few larger ones. The structure of sections also differs among chapters. However, I feel this arrangement is to the benefit of the reader and allows the progression of ideas within and among different chapters and sections to be followed more easily.

CHAPTER 1

INTRODUCTION

1.1 SEA TURTLE POPULATIONS OF EASTERN AUSTRALIA

There are 2 extant families of sea turtles in the world, represented by seven species. The family Cheloniidae contains *Caretta caretta* (loggerhead), *Chelonia mydas* (green), *Eretmochelys imbricata* (hawksbill), *Lepidochelys kempii* (Kemp's ridley), *Lepidochelys olivacea* (olive ridley) and *Natator depressus* (flatback) while the family Dermochelyidae contains only *Dermochelys coriacea* (leatherback). All except the Kemp's ridley occur in Australian waters. Legislative protection of these animals by International, National (Australia) and State (Queensland) bodies is summarised in Table 1.1.1.

Global sea turtle populations have been greatly reduced due to human activity, including increased exploitation of marine and coastal waters (e.g. commercial fishing), beach development, and harvesting of eggs, juveniles and adults (Lutcavage *et al.* 1996). Australian herds of green, loggerhead and hawksbill turtles are of global significance (Limpus 1982, 1992, Limpus and Reed 1985a, Limpus and Parmenter 1986, Groombridge and Luxmore 1989, Guinea 1994, Limpus and Reimer 1994, Miller 1994, Prince 1994) with Australian nesting populations possibly the largest remaining in the world (Limpus 1980a, 1982, Limpus *et al.* 1983c, 1992, Gyuris and Limpus 1988, Miller and Limpus 1991). The flatback turtle is endemic to the Australian continental shelf (Limpus *et al.* 1981, 1983a, 1983b, 1989, Limpus 1982, Parmenter 1993, 1994a) and has only been observed nesting in Australia (Limpus *et al.* 1981, 1983a, 1983b, 1989, Parmenter 1993).

Table 1.1.1 Protection of sea turtles by International, National and State Legislation.

Turtle	1994 IUCN Red List (International)	CITES Listing (International)	Commonwealth Endangered Species Act 1992 (Australia)	Queensland Nature Conservation Act 1992 (Queensland)
Loggerhead	EN	Appendix 1	EN	Endangered*
Green	CR	Appendix I/r- Cuba	VU	
Leatherback	EN	Appendix I/r- Suriname	VU	
Hawksbill	CR	Appendix I/r- Cuba	VU	Threatened
Kemp's Ridley	CR	Appendix I	-	
Olive Ridley	EN	Appendix I/w- Japan	EN	
Flatback	VU	Appendix I	VU	

CR- Critically Endangered; EN- Endangered; VU- Vulnerable; r- reservations; w- reservations withdrawn
*Reclassified as Endangered by decree of Governor in Council from its 1992 classification of Threatened
(Sources: Commonwealth Endangered Species Act 1992, Queensland Nature Conservation Act 1992, IUCN (World Conservation Union) Red List of Threatened Animals 1994, CITES Appendix I 1998)

The status of resident leatherback and olive ridley turtles has yet to be comprehensively investigated (Limpus 1975, Limpus and McLachlan 1979, 1994, Harris 1994) since observations of nestings are both temporally and spatially sporadic, the majority being single emergences of different females (Cogger and Lindner 1969, Limpus and McLachlan 1979, 1994, Limpus 1982, Limpus *et al.* 1983a, 1984a). Descriptions of Queensland feeding grounds, nesting sites and pertinent publications are summarised in Tables 1.1.2 and 1.1.3.

The reproductive migrations of sea turtles (Bustard and Limpus 1970, Limpus *et al.* 1983b, 1992, Parmenter 1983) requires management and protection of Australian populations to be viewed in both a national and international legislative context. Sexually mature animals preparing to breed migrate from their feeding grounds to the courtship areas and nesting beaches (that are presumably their area of natal origin), and return at the conclusion of an individual's reproductive season (Limpus 1989). Migrations of greater than 2,500km have been recorded (Limpus *et al.* 1992). The presumed pelagic dispersal phase of hatchling and post-hatchling turtles may also involve great distances (Limpus 1985, 1992, Miller 1994), with the exception of *N. depressus* hatchlings which remain over the Australian continental shelf (Walker and Parmenter 1990, Walker 1991a, 1994).

Table 1.1.2 Major sea turtle nesting sites and feeding areas in, and adjacent to, eastern Australia.

Turtle	Nesting Sites	Feeding Grounds
Loggerhead	1. Capricorn-Bunker Group of Islands of sGBR 2. Bundaberg to Round Hill Head Coastline 3. Swain Reefs of sGBR	Entire Queensland east coast including reef, shallow bays and estuaries; eGoC; PNG
Green	1. Raine Is.-Pandora Cay of nGBR 2. Capricorn-Bunker Group Islands of sGBR 3. Wellesley Group of sGoC	1. nGBR breeding stock- Torres Strait, sPNG, southern Cape York Peninsula, Northern Territory, Aru Is. (Indonesia) 2. sGBR breeding stock- Coral Sea perimeter including PNG and New Caledonia 3. Wellesley Group- unknown
Leatherback	Nesting has only been observed from Bundaberg and coastline 160km north	Coastal waters from south Queensland to central New South Wales
Hawksbill	1. islands of central to eastern Torres Strait 2. inner shelf islands of nGBR	sGBR, nGBR; Torres Strait reefs
Olive Ridley	No major rookeries have been discovered, however low density nesting occurs around Crab Island in GoC	Unknown
Flatback	1. Crab Is. 2. Peak Is. 3. Wild Duck and Avoid Islands	GoC, Torres Strait and shallow coastal waters protected by the GBR

s- southern; n- northern; GBR–Great Barrier Reef; GoC- Gulf of Carpentaria; PNG- Papua New Guinea
(Limpus 1982, 1994)

Table 1.1.3. Synopsis of recent publications concerning Queensland sea turtles.

Turtle	Feeding Grounds/Populations	Developmental and Breeding Migration	Breeding Grounds/Populations	Genetic Studies	Population/Growth Modelling
Loggerhead	Limpus *et al.* 1994	Limpus 1985, Limpus *et al.* 1992	Limpus 1982, Limpus and Reed 1985b, Limpus and Reimer 1994	Gyuris and Limpus 1988, Harry and Briscoe 1988, FitzSimmons *et al.* 1995	Somers 1994, Heppell *et al.* 1996
Green	Limpus *et al.* 1993a, Limpus and Reed 1985a	Limpus *et al.* 1992	Limpus 1982, Limpus and Nicholls 1988, Limpus 1993a, Limpus *et al.* 1993a, Limpus and Nicholls 1994	FitzSimmons 1997, FitzSimmons *et al.* 1995, FitzSimmons 1998	Limpus and Chaloupka 1997
Leatherback	Limpus and McLachlan 1979, 1994	Limpus and McLachlan 1994	Limpus and McLachlan 1979, Limpus *et al.* 1984a		
Hawksbill	Limpus 1992, Miller 1994	Limpus 1992, Miller 1994, Miller *et al.* 1998	Limpus 1980a, Limpus *et al.*, 1983c, Limpus *et al.* 1993b, Miller 1994, Loop *et al.* 1995	Broderick *et al.* 1994	Chaloupka and Limpus 1997
Olive Ridley	Limpus 1992, Harris 1994		Harris 1994		
Flatback	Parmenter 1994a	Limpus *et al.* 1983a, Walker and Parmenter 1990, Walker 1991a, 1994, Parmenter 1994	Limpus *et al.* 1981, Limpus *et al.* 1983a, Limpus *et al.* 1984b, Parmenter 1994, Parmenter and Limpus 1995	FitzSimmons *et al.* 1995	

Limpus and Reimer (1994) reported an alarming decline in nesting populations of the loggerhead turtle in Queensland (50-80% over 10-18 years dependent upon study site). Although loggerhead turtles are found worldwide in both tropical and temperate waters, there are very few major breeding aggregations where thousands of females nest annually (Limpus and Reimer 1994). These are limited to Oman in the Indian Ocean, eastern USA in the Atlantic Ocean, Greece and Turkey in the Mediterranean Sea, and south Queensland and southern Japan in the Pacific Ocean (Dodd 1988).

Loggerhead nesting in the south Pacific Ocean occurs almost entirely on the southern Great Barrier Reef and Bundaberg coastline (Limpus 1985), so the population decline reported by Limpus and Reimer (1994) is of serious concern. Increased mortality leading to catastrophic population decline is most likely due to incidental capture of turtles in the northern and eastern Australian fishing industry, with harvest for food, boat strike, ingestion of discarded debris and mainland fox depredation of nests also contributing to the losses (Limpus and Reimer 1994).

Mitochondrial DNA analysis has shown there is no evidence of genetic interchange between loggerhead populations of the northern and southern Pacific Ocean basins, so it is unlikely depleted populations would be restocked from another area within a reasonable time frame (Bowen *et al.* 1994). The loss of turtles is further compounded by high nest mortality in the southern Great Barrier Reef coral cays compared to mainland rookeries (Limpus *et al.* 1983d). The Capricorn-Bunker islands (where male hatchlings are predominantly produced) and adjacent mainland rookeries (where female hatchlings are predominantly produced) form a single

breeding unit (Limpus *et al.* 1983d, 1985) so depletion of breeding stock from one location has serious ramifications for the other.

On coral cays the hatch success of loggerhead turtle nests is also low compared with adjacent green sea turtle nests. The appearance of fungi in high mortality nests was believed to be a contributing factor (Limpus *et al.* 1983d), though prior to this study the role of fungi as a nest contaminant or infectant of viable eggs had not been rigorously investigated.

The current rate of decline of Queensland loggerhead turtles suggests that nightly nesting may soon not occur, even at the peak of the season, at major rookeries such at Mon Repos (Limpus and Reimer 1994). This necessitates every anthropogenic and natural cause of mortality be investigated, in order to maximise hatch success at all rookeries and increase survivorship of all age-classes.

The aim of this study was to identify the fungi invading failed eggs, determine their source(s) and factors influencing their presence in turtle nests, and resolve whether their appearance was post-mortem, or contributed directly to embryo mortality.

1.2 THE SEA TURTLE EGG, EMBRYO AND NEST ENVIRONMENT

Conditions within the sea turtle nest have the potential to influence egg survival, embryonic growth, neonate size and weight, and subsequent hatchling performance. To understand the relevance of fungal invasion during incubation, a

knowledge of egg structure and components, requirements for embryogenesis and the nest environment is necessary.

1.2.1 The Cleidoic/Amniotic Egg

Adaptations of the ancestral anamniotic egg to enhance the efficiency of terrestrial reproduction have included the production of an eggshell that provides mechanical support whilst still allowing the exchange of oxygen, carbon dioxide and water; the presence of shell membranes (lining the inner surface of the shell) and water-rich albumen to prevent desiccation; and the inclusion of a large, concentrated nutrient source (principally the yolk) (Romer 1957, Pough *et al.* 1996, Gerhart and Kirschner 1997). This has resulted in sea turtle embryonic development occurring within a closed (cleidoic) system, wherein only respiratory gases, water vapour and heat are readily exchanged with the external environment.

Development of the general cleidoic egg was complemented by the evolution of morphological changes to the embryo. Four extraembryonic membranes (the yolk sac, amnion, chorion and allantois) developed as major outgrowths from the body of the embryo (Romer 1957, Freeman and Vince 1974, Pough *et al.* 1996, Gerhart and Kirschner 1997).

Formation of the extraembryonic membranes in sea turtles occurs very early in development, at mid to late gastrulation, after oviposition (Gerhart and Kirschner 1997). The yolk sac (which later in development becomes an extension of the small intestine) spreads out from the embryo body so endodermal tissue eventually envelops the entire yolk. Blood vessels in the yolk sac tissue become responsible for

the uptake of yolk nutrients and their transfer to the embryo (Freeman and Vince 1974, Noble and Cocchi 1990, Pough *et al.* 1996). The amnion and chorion are derived from ectoderm which begins to expand from the blastodisc edges at the time of gastrulation. The amnion surrounds the body of the embryo and provides a supportive, fluid-filled environment for development (Freeman and Vince 1974, Gerhart and Kirschner 1997), while the chorion lines the inner surface of the eggshell and eventually surrounds the entire embryo/yolksac complex (Freeman and Vince 1974, Gerhart and Kirschner 1997). The allantois, which develops as an outgrowth of the hind gut posterior to the yolk sac and lies within the chorion, is used for storing nitrogenous waste. Later in development, the chorion and allantois fuse to become the chorioallantoic membrane, which transports respiratory gases between the shell and embryonic circulation (Freeman and Vince 1974).

1.2.2 Egg Components

All sea turtles lay white, spherical eggs (Miller 1985), although egg size may vary among clutches laid by females of the same species, and among species (Hirth 1980, van Buskirk and Crowder 1994). Table 1.2.1 summarises the known reproductive characteristics of sea turtles nesting in eastern Australia.

In sea turtles the eggshell contributes little to the weight of the egg, while proportions of yolk and albumen appear to differ between species (Table 1.2.2). Variation may be due to differences in water movement between the egg components prior to oviposition, or the time at which they were analysed (Miller 1985).

Table 1.2.1. Reproductive characteristics of eastern Australian sea turtles. Mean±SD (no. of clutches).

Turtle	Remigration Interval (years)	Clutches/Season	Renesting Interval (days)	Clutch Count	Egg Weight (g)+	Egg Diameter (cm)+	Rookery	Source
Loggerhead	-	-	14.51±3.48(37)	124.37±20.07(27)	39.22±3.04(17)	4.120±0.119(17)	Heron Is. 1980-81	Limpus *et al.* 1984c.
Green	-	-	13.520±0.110(253)	112.000±21.56 (35)	50.02±7.31(11)	4.46±0.18(22)	Heron Is. 1974-75	Limpus 1980b
			14.080±1.646(264)	115.240±27.88 (50)	46.930±5.251 (16)	4.407±0.143(16)	Heron Is. 1980-81	Limpus *et al.* 1984c
Leatherback	-	Up to 3	-	82.79±13.09(14)	81.95±4.24(7)	5.320±0.105(12)	south QLD 1974-82	Limpus *et al.* 1984a
			9.700±0.750(6)	86.07±15.74(16)	82.21±4.15(8)	5.330±0.112(13)	south QLD 1974-91	Limpus and MacLachlan 1994
Hawksbill	-	2.60±1.30(363)	14.30±0.100(427)	124.10±26.00 (310)	25.70±2.55(103)	3.48±0.16(107)	Milman Is.	Loop *et al.* 1995
			-	111.70±19.95(29)	26.40±1.93(6)	3.64±0.130(7)	nGBR	Limpus 1980a
Olive Ridley	-	-	-	109.0(1)	35.700±0.747(1)	3.68±0.041(1)	Crab Is.	Limpus *et al.* 1983a
Flatback	2.650±0.920(40)	2.84±0.78(43)	16.00±1.890(115)	-	-	-	Bundaberg coast	Limpus *et al.* 1984b
			-	50.2±10.7(87)	77.80±4.66(87)	5.21±0.105(87)	south QLD	Limpus 1971
	2.225±0.770(889)		-	54.520±9.950(44)	-	-	Peak Is. 1980	Limpus *et al.* 1981
	-		-	53.352±9.750 (409)	-	-	Peak Is. 1980-86	Parmenter and Limpus 1995

+n- no. of clutches with 10 eggs per clutch sampled
nGBR- northern Great Barrier Reef; QLD- Queensland
- indicates no data available

Table 1.2.2. Relative proportions of egg components.

Turtle	Average Egg Wt (g)	Percentage of Fresh Egg Weight			Source
		Shell	Albumen	Yolk	
Loggerhead	34.4	5.8	39.2	55.0	Tomita 1929*
	34.7	4.8	45.5	49.2	Miller 1982
Green	51.3	4.3	28.4	67.3	Richard and Nguyen 1961*
Leatherback	71.8	4.3	48.9	46.8	Simkiss 1962
Flatback	74.0	5.2	45.2	49.6	Hewavisenthi 1999

*Cited by Ewert 1979 and Miller 1985

The egg yolk is the primary nutritional source for the developing turtle embryo (Ewert 1979), containing all of the lipid and most (or all) of the protein required for embryonic metabolism and growth (Congdon *et al.* 1983). It also supplies some of the calcium required during osteogenesis (Packard and Packard 1986). Eggs of loggerhead, green, hawksbill, leatherback (Seaborn and Moore 1994) and flatback (Hewavisenthi 1999) turtles each have distinctive fatty acid profiles, which probably reflect the adult diets of each species (Ackman *et al.* 1971, Joseph *et al.* 1985). Residual yolk supports neonate metabolic demands during hatching, nest emergence and initial swimming away from the nesting beach (Kraemer and Bennett 1981).

Within the egg, the yolk is surrounded by albumen which prevents it from directly contacting the eggshell membranes. Albumen consists mostly of proteins with trace quantities of carbohydrates and lipids (Palmer and Guillette 1991), but it primarily acts as a reservoir for water, most of which is transferred into the vitelline sac during the first week of incubation (Morris *et al.* 1983, Packard *et al.* 1981, 1983). After this, the albumen is present only as a thin, rubbery layer beneath the shell membrane (Packard 1999).

Reptilian albumen proteins may be nutritive, supportive or hydrophilic. It maintains a cushioned, homeostatic environment for embryonic development, while also providing protection against microbial invasion (as summarised by Palmer and Guillette 1991).

The eggshell separates the developing embryo from the external environment while still allowing the transfer of heat, moisture and respiratory gases required for embryogenesis. The flexible shells of sea turtles eggs consist of a well-defined calcareous layer approximately the same thickness as the adjacent shell membrane (Ewert 1979, Packard 1980, Solomon and Baird 1979). The calcareous component of reptilian eggshells is calcium carbonate, present as the aragonite crystal form in chelonian eggs (Young 1950, Baird and Solomon 1979, Packard and Packard 1979, Packard 1980), although calcite crystals have been reported in the shells of eggs produced by farmed green sea turtles (Solomon and Baird 1980).

1.2.3 Fecundity

Female sea turtles lay multiple clutches during a single breeding season. They rarely nest in successive years (Hirth 1980, van Buskirk and Crowder 1994), with the exception of the flatback turtle (C.J. Parmenter pers.comm.). Reproductive characteristics of eastern Australian sea turtles have been summarised in Table 1.2.1.

1.2.4 Ovulation, Fertilisation and Shelling

The sea turtle ovary and the 5 zones of the oviduct (the infundibulum, aglandular segment, magnum or albumen forming segment, uterus or shell forming segment and vagina) have been described by Aitken and Solomon (1976a, b). Ovulation occurs within 36hr of the previous oviposition (Licht 1980) and ova enter the oviduct via the infundibulum. Fertilisation occurs in the magnum (Solomon and Baird 1979) from sperm stored in sperm storage tubules within the magnum (Solomon and Baird 1979, Gist and Jones 1987, Gist and Congdon 1998, Gist and Jones 1989, Gist *et al.* 1990) and less frequently at the utero-vaginal junction (Solomon and Baird 1979, Gist and Congdon 1998) of the oviduct. While single paternity of individual and successive clutches appears prevalent, there is potential for multiple paternity (Table 1.2.3).

Table 1.2.3. Evidence for multiple paternity in sea turtles clutches.

Turtle	Clutches with Multiple Paternity (n= # clutches)	Rookery	Source
Loggerhead	36.36% (22)	Mon Repos, Australia	Harry and Briscoe 1988
	33.33% (3)	-	Bollmer *et al.* 1999
	21.05% (38)	Melbourne Beach, Florida	Moore and Ball 1998
Green	9.09% (22)	sGBR, Australia	FitzSimmons 1997, 1998
Leatherback	0.00% (4)	Playa Grande, Costa Rica	Rieder *et al.* 1998
	9.09% (11)	Caribbean coast of Costa Rica	Curtis *et al.* 1998
	9.09% (22)	St Croix, U.S. Virgin Islands	Dutton *et al.* 1998
Kemp's Ridley	53.85% (26)	Rancho Nuevo, Mexico	Kichler *et al.* 1999

As embryogenesis commences, the embryo on its yolk travels through the magnum where albumen is secreted by specialised cells lining the anterior glandular region (Aitken and Solomon 1976b). The caudal shell forming segment of the oviduct (the uterus) appears to secrete both the protein-carbohydrate shell membrane and the calcium of the shell matrix (Solomon and Baird 1976, 1979). During shell formation the chelating power of the eggshell matrix must be strong enough to remove calcium ions from the blood, yet weak enough for disruption by carbonate ions, thus allowing calcium to precipitate as a carbonate (Simkiss and Tyler 1959).

Eggs of the green (Miller 1982) and olive ridley (Owens 1980) sea turtles are contained in a slightly calcified shell membrane within 3 days of ovulation. Shell thickness then increases rapidly until 8-9 days post-nesting when calcification is complete and the egg is structurally ready for oviposition. However, at the completion of calcification, embryonic development has only reached early gastrulation and needs to attain mid to late gastrulation to maximise post-ovipositional survival (Miller 1982).

1.2.5 Oviposition

Oviposition usually occurs at night and the general nesting process is similar in all sea turtle species (Hendrickson 1982). Eggs are deposited singly or in groups of 2-3 (occasionally 4) from both oviducts simultaneously (Miller 1996). At the completion of egg laying, the nest is covered with sand which provides thermal and hydric insulation.

Prior to oviposition, embryonic development is suspended at gastrulation. The trigger(s) for commencement of further development is unknown, but possibilities include lack of movement, thermal shock, oxygen availability, hormonal influence or water uptake. Within 6-18hrs of oviposition the extraembryonic membranes have attached to the inner shell membrane (Miller 1985) and the egg becomes sensitive to movement for the early stages of development (Limpus *et al.* 1979, Parmenter 1980). Embryonic diapause may be maintained by temperature depression to 7-10°C (Harry and Limpus 1989) for up to 48hrs (pers.ob.) if transport for artificial incubation is required.

1.2.6 Post-oviposition Development

Normally developing eggs exhibit a "white-spot" that expands across the eggshell, closely paralleling the internal development of the chorioallantois (Thompson 1985). Its chalk-white appearance initially reflects structural and physical changes to the eggshell associated with drying (Webb *et al.* 1987). This colouration is maintained throughout incubation, and is reflective of the eggshell's ability to meet the increasing respiratory gas exchange demands (Lomholt 1976, Rahn *et al.* 1979).

Eggs that do not develop a white spot have often been classified as infertile (Bustard 1972, Fowler 1979, Stancyk *et al.* 1980, Whitmore and Dutton 1985), but the delay between egg mortality and excavation of the emerged nest to inspect unhatched eggs may result in difficulty distinguishing between intraoviductal/early embryonic death (before the formation of blood isles) and subsequent decomposition from "infertility" (Miller 1985).

Miller (1985) has defined 25 stages of embryonic development between oviposition and hatching (of 31 in total). All species are morphologically similar until Stage 22, and divergence only becomes apparent beyond this stage (Miller 1985).

In addition to providing a physical barrier, the eggshell also acts as a calcium supply for the developing embryo. Calcium is the major inorganic constituent of olive ridley turtle eggshell at oviposition (21.08%) with only trace amounts of magnesium (0.056%), sulphur (1.143%) and potassium (0.049%) present (Sahoo *et al.* 1998). Eggshell calcium provides 60% of olive ridley (Sahoo *et al.* 1998), 75% of leatherback (Simkiss 1962) and 62% of green and loggerhead (Bustard *et al.* 1969) requirements for ossification of the embryonic skeleton. The majority of this is required in the second half of incubation, when the chorioallantois mediates calcium extraction from the eggshell and subsequent transport to the embryonic vasculature (Packard and Clark 1996).

1.2.7 Nest Environment

1.2.7.1 Thermal Micro-climate

Sea turtle embryos only develop between a lower limit of 25-27°C and an upper limit of 33-35°C (Ackerman 1996). Sexual differentiation of loggerhead (Yntema and Mrosovsky 1980, Limpus *et al.* 1985, National Research Council 1990), green (Miller and Limpus 1981), leatherback (Rimblot *et al.* 1985, Binckley *et al.* 1998), hawksbill (Mrosovsky *et al.* 1992, Godfrey *et al.* 1999), olive ridley (Ruiz *et al.* 1981 in Miller 1996) and flatback (Hewavisenthi 1999) turtles is determined by temperature. Cooler temperatures produce males and warmer

temperatures females, although the threshold or pivotal temperature, where 50% each of males and females are produced, varies among populations and species (Limpus *et al.* 1985).

The proportion of embryonic development occurring above the threshold temperature, and not the mean daily temperature, determines hatchling sex ratio (Georges *et al.* 1994). The critical period for sex determination occurs during the middle third to middle half of incubation, which is known as the thermosensitive period (TSP), when embryonic sex may be irreversibly influenced by temperature (Yntema 1979, Bull and Vogt 1981, Pieau and Dorizzi 1981, Yntema and Mrosovsky 1982). The TSP usually encompasses Stages 22-27 (Yntema and Mrosovsky 1982, Mohanty-Hejmadi and Dimond 1986, Desvages *et al.* 1993).

During the TSP ovaries differentiate if high levels of endogenous oestrogens have been synthesised from androgens by the enzyme aromatase. Lower aromatase activities, and thus oestrogen levels, allow development of the testes. While the pathway for the influence of temperature during sex determination has not been definitively demonstrated, three broad possible mechanisms have been proposed:

- temperature activates or suppresses synthesis of a feminising factor or masculinising factor
- temperature activates or suppresses heat shock proteins involved in oestrogen binding to oestrogen receptors
- temperature is involved in the dissociation of heat shock proteins from the oestrogen-oestrogen receptor complex which is then activated (Pieau 1996, Pieau *et al.* 1999).

Other incubation parameters affected by temperature include incubation period (Hendrickson 1958, Bustard and Greenham 1968, Mrosovsky and Yntema 1980) and hatching success (Bustard and Greenham 1968, Bustard 1972, McGehee 1979, Miller and Limpus 1981). Nutrient mobilisation and resulting hatchling size of freshwater turtle embryos are also related to thermal conditions during incubation (Gutzke *et al.* 1987, Packard *et al.* 1987).

1.2.7.2 Hydric Micro-climate

Very little is known about the influence of the hydric environment on sea turtle nests. Whilst sea turtle eggs contain a supply of water in their albumen at oviposition (Ackerman 1996), this may be supplemented by water gained through the transformation of yolk (Ar and Rahn 1980) and the exchange of water vapour and possibly liquid water with the environment (Ackerman *et al.* 1985, Kam and Ackerman 1990, Ackerman 1991). Eggs laid in moist substrates absorb water soon after oviposition to become turgid (Miller 1985) but lose water near the end of incubation (Bustard and Greenham 1968, Kraemer and Richardson 1979).

Sea turtle eggs are very sensitive to desiccation (Kaufman 1968 in Miller 1996) and inundation (Ragotzkie 1959, Kraemer and Bell 1980). Their embryonic growth appears to be independent of water exchange within the range –10 to +30% of initial egg mass (Ackerman 1996) with hatching only adversely affected below –40% (Miller 1996). Excessive rainfall or tidal inundation may suffocate developing embryos and hatchlings still in the nest (Kraemer and Bell 1980).

Hewavisenthi and Parmenter (2001) found that total egg water exchange influenced embryonic nutrient mobilisation, hatchling size and hatchling energy reserves in flatback turtles. Studies on freshwater turtles have demonstrated water availability has an effect on nutrient mobilisation of yolk (Gutzke *et al.* 1987, Packard *et al.* 1988), incubation period (Packard *et al.* 1981, Morris *et al.* 1983, Gutzke *et al.* 1987, Packard *et al.* 1987), hatching success (Packard *et al.* 1981, Bobyn and Brooks 1994, Cagle *et al.* 1993) and hatchling size (Gutzke *et al.* 1987, Packard *et al.* 1987, Brooks *et al.* 1991). Hatchling size has the potential to influence locomotor performance, subsequent growth, survivorship, and ultimately size at first reproduction and therefore fecundity (as reviewed by Miller and Packard 1992).

1.2.7.3 Gas Exchange

Respiratory gases are only able to move freely between the beach surface and turtle nest through the gas-filled fraction of the sand, but this is usually less than half of the sand volume. Gas exchange within the clutch can only occur through the air spaces among the eggs (Ackerman 1996).

Respiratory gas exchange must occur by convection or diffusion. Convection through sand may be induced by temperature gradients, changes in atmospheric pressure (Ackerman 1996), or displacement of soil air by water table movement (see Prange and Ackerman 1974, Maloney *et al.* 1990). The magnitude of convection in sand covering the turtle nest is thought to be very small (Koorevaar *et al.* 1983 in Ackerman 1996), so diffusion is likely to be primarily responsible for gas exchange (Prange and Ackerman 1974).

For net diffusion to occur, concentration differences of respiratory gases must occur between the core and periphery of the egg mass, and also between sand at the edge of the clutch and the beach surface. This may be complicated in areas of high clutch density, since nests within 1m of each other may influence their neighbour's concentration gradient. When this occurs, gas exchange through the top or bottom of the nest may become more important than through the sides (Ackerman 1996).

Gas exchange by sea turtle clutches has been modelled by Ackerman (1977) and Maloney *et al.* (1990). Metabolic activity of the embryo is dependent on the background concentrations of respiratory gases (in addition to thermal and hydric conditions). Dehydration of the eggshell soon after oviposition is essential for establishment of a gaseous connection between the nest atmosphere and respiratory exchange surfaces within the egg itself (Lomholt 1976, Rahn *et al.* 1979). Oxygen must pass across the eggshell and associated eggshell membrane (Ackerman and Prange 1972, Deeming and Thompson 1991) before it can be transported by the chorioallantoic capillaries to the embryonic tissue.

The pattern of oxygen uptake by loggerhead and green turtle eggs is a sigmoidal increase throughout incubation (Ackerman 1981a). It parallels embryonic growth (Ackerman 1981b) with the maximum oxygen consumption occurring during growth stages rather than differentiation. Oxygen consumption rises sharply after the first half of incubation but slows prior to hatching (Ackerman 1981b, Thompson 1993), as does growth (Ackerman 1981a).

Adequate gas exchange is typical of most sea turtle nests (Prange and Ackerman 1974, Ackerman 1980), although it may be adversely affected by substrate particle size and sand water content (Ragotzkie 1959, Prange and Ackerman 1974, Ackerman 1980, Kraemer and Bell 1980, Ackerman 1981b). Inadequate gas exchange slows embryonic growth and may cause mortality (Ackerman 1981b).

1.2.8 Embryonic Metabolism and Growth

During embryonic growth, lipids are moved from the yolk to embryonic cells where they are metabolised and used for differentiation and growth (Ackerman 1996). Lipid mobilisation may be influenced by nest temperature (Gutzke *et al.* 1987, Packard *et al.* 1987) and moisture (Gutzke *et al.* 1987, Packard *et al.* 1988).

Stage-based embryonic growth and development for loggerhead, green, leatherback, hawksbill and flatback turtles have been described by Miller (1982).

1.2.9 Nitrogen Excretion

Most embryonic turtles detoxify the ammonia released in protein catabolism by converting it to soluble urea which is then stored in the allantois (Packard and Packard 1983, Packard *et al.* 1983, 1984, 1985). A portion of the limited water reserve must, therefore, act as a solvent for this metabolic waste (Packard and Packard 1983).

Embryos incubating in wetter environments catabolise larger quantities of protein (Packard *et al.* 1988) so the total amount of urea increases rapidly (Packard *et al.* 1984). To offset this, more environmental water may be absorbed by the egg to

dilute urea concentrations (Morris *et al.* 1983, Packard *et al.* 1983). Eggs experiencing relatively dry conditions have slower metabolic rates (Packard *et al.* 1988) and produce less total urea, but its concentration is higher (Packard *et al.* 1984). Concentrations approach those known to inhibit enzyme activity in other vertebrates (Hand and Somero 1982 in Packard and Packard 1989, 1983) but do not appear to adversely affect hatchling size or the mass of residual yolk (Packard and Packard 1989).

1.2.10 Incubation Period

As well as influencing hatchling sex, nest temperature also determines incubation duration: there is an inverse relationship between incubation period and temperature. At 23-25°C *C. mydas* eggs have an incubation period of 94 days (Packard and Packard 1988) whilst at 32°C incubation hatching occurs after only 49 days (Bustard and Greenham 1968). Eggs that experience temperatures lower than 23°C in the last trimester rarely hatch (Miller 1985). Natural beach nests usually experience temperatures of 26-32°C, but short periods may be spent outside this range (Bustard 1972, Ewert 1979). Within the range of 26-32°C, a 1°C alteration lengthens or shortens the incubation period by approximately 5 days (Mrosovsky 1980).

Incubation duration may also be affected by substrate water potential, with eggs taking 2-4 days longer to hatch on wet substrates (*ca.* –150 kPa) than on dry (*ca.* –900 kPa) (reviewed by Steyermark 1999). Average incubation periods in natural nests for eastern Australian turtle rookeries are included in Table 1.2.4.

Table 1.2.4. Productivity of sea turtle nests in eastern Australia. Mean±SD (no. of clutches).

Turtle	Incubation Period[#] (days)	Hatchling Carapace Length (cm)	Hatchling Weight (g)	Hatch Success[^] %	Rookery	Source
Loggerhead	-	4.368±0.154(13)	19.318±1.181(13)	-	Heron Is. 1980-81	Limpus *et al.* 1984c
	-	-	-	72.4±28.0(17)	Heron I. 1980-81	Limpus *et al.* 1983d
	-	-	-	90.7±8.4(60)	Mon Repos 1980-81	Limpus *et al.* 1983d
Green	-	4.97±0.19(11)	24.83±1.84(11)	-	Heron Is. 1974-75	Limpus 1980b
	-	4.89±0.188(22)	24.93±2.360(22)	-	Heron Is. 1980-81	Limpus *et al.* 1984c
	-	-	-	92.0±7.3(60)	Heron Is. 1980-81	Limpus *et al.* 1983d
Leatherback	60.5±0.71(2)	-	-	-	South QLD 1975-76	Limpus and MacLachlan 1979
	-	5.88±0.292(4)	46.86±4.659(4)	15.3±17.6(7)	South QLD 1974-82	Limpus *et al.* 1984a
Hawksbill	56.9±3.3(17)	3.96±0.21(17)	13.3±1.64(17)	79.9±11.8(13)[*]	Milman Is.	Loop *et al.* 1995
	-	4.12±1.38(26)	14.6±1.07(11)	-	nGBR	Limpus 1980a
Olive Ridley	-	-	-	-	-	-
Flatback	53.4(15)	-	-	74.64±19.94(215)[*]	Peak Is. 1980-1986	Parmenter and Limpus 1995
	-	6.12(19)	43.6(19)	80-90%	South-east QLD	Limpus 1971
	-	6.31±3.31(10)	41.14±3.31(10)	83.1±18.66(136)	Peak Is. 1995-97	Hewavisenthi 1999

[#]Incubation Period- time from oviposition until hatchling emergence from nest
[^]Hatch Success=(# number of eggs hatched/clutch count) x 100%
[*]Emergence Success=((number of eggs hatched – number of hatchlings not reaching nest surface)/clutch count) x 100%
- indicates no data available

1.2.11 Hatching and Hatch Success

Hatching synchrony is important in enabling hatchlings to dig their way to the beach surface (Hendrickson 1958, Carr and Hirth 1961). Different eggs within a single reptilian nest may experience varied temperature regimes if the nest is located within a zone of diurnal temperature variation (Thompson 1988). Since diurnal temperature fluctuation is only apparent at depths less than 50cm (Hillel 1980), and nests of Australian sea turtles are generally deeper than this, developmental asynchrony within a single nest is unlikely (Thompson 1989). The metabolically induced thermal differences within sea turtle nests (Maloney *et al.* 1990, Godfrey *et al.* 1997) are unlikely to have a dramatic effect on the rate of embryonic development because they occur in the last third of incubation (Maloney *et al.* 1990) when growth rate is least influenced by thermal variation (Yntema 1978).

There is a volumetric reduction in nest contents 7-8 days prior to emergence (Kraemer and Richardson 1979) as water evaporates due to increased vapour pressure within the egg caused by metabolic heat (Packard *et al.* 1977). As eggs lose turgor, the shell closely contacts the embryo and pre-hatching movements are easily detected by turtles in adjacent eggs which facilitates synchronised hatching (Bustard 1972). When ready to hatch, embryos use their caruncle to tear the amniotic and chorioallantoic membranes and the eggshell (pipping). This process may be made easier by the reduction in egg turgor prior to pipping (Bustard and Greenham 1968) since it brings the embryo into close contact with the shell (Kraemer and Richardson 1979). Exfoliation/flaking of the eggshell in the week prior to hatching (Miller 1982) results from calcium uptake by the rapidly maturing embryo (Simkiss 1962), weakening the eggshell and allowing easier pipping.

At pipping, albumen, allantoic fluids and amniotic fluids drain from the torn egg, allowing space for the neonate to free itself from the eggshell (hatching) (Miller 1996). Residual yolk is internalised over several days (Bustard 1972) to fuel emergence from the nest, crawling across the beach, and the initial swim (Kraemer and Bennett 1981). Formation of a space above the nest contents due to loss of egg turgor and near-simultaneous pipping and hatching of the entire clutch (Kraemer and Richardson 1979) allows a greater volume within which hatchlings can move and extricate themselves from the shell mass and subsequently dig toward the beach surface (Miller 1996).

Hatch success is usually above 80% (National Research Council 1990) when clutches are unaffected by external factors such as erosion, depredation, plant root invasion, excessive rainfall, tidal inundation and microbial infection. The hatch success of nests at eastern Australian rookeries is included in Table 1.1.4. Unhatched eggs from emerged nests may be opened and the contents categorised as: undeveloped (nil visible embryonic growth); early embryonic death (the presence of blood but no visible embryo); or a carcass able to be identified to embryonic stage (Miller 1985).

1.2.12 Hatchling Emergence

Early pipping neonates are quiescent until the rest of the clutch pips (Carr and Hirth 1961) and all remain in the nest cavity for a short period before digging out (Daniel and Smith 1947). Under normal circumstances, however, hatchlings do not delay digging for more than 3 days (Kraemer and Richardson 1979).

Hatchlings are usually able to dig from the shell mass to the sand surface within a day (Balazs and Ross 1974), although cyclonic events may deposit additional sand on the nest and delay emergence (e.g. Bell and Hallam (1999) report eventual emergences from nests of 2.4m in depth). Mutual stimulation of adjacent animals (social facilitation- Carr and Hirth 1961) ensures a number of animals dig upwards simultaneously and scratch sand away from the nest plug above so it sifts down and settles beneath them (Miller 1996). Once near the surface the hatchlings usually pause in the last few centimetres of sand (Bustard 1967), often with their heads partially exposed (Bustard 1967, Dial 1987), waiting for favourable conditions before leaving the protection of the nest.

Emergence usually occurs at night (Hendrickson 1958, Bustard 1967, Neville *et al.* 1988, Hays *et al.* 1992), although it has been observed in the early morning (Chavez *et al.* 1968 in Witherington *et al.* 1990) and late afternoon (Witzell and Banner 1980). Emergence may also occur at midday during overcast conditions (pers.ob.). The majority of hatchlings from a single nest usually emerge within minutes of each other, but individuals or small groups may precede or follow the main group (Witherington *et al.* 1990).

Three hypotheses, all of which are temperature related, have been proposed regarding the timing of emergence:

- Hatchlings emerge after the surrounding sand temperature falls below a critical threshold (Hendrickson 1958, Mrosovsky 1968, Bustard 1972);
- Hatchlings are negatively thermotaxic (Gyuris 1993) and emerge when a negative thermal gradient develops in the sand column above the nest

(Moran *et al.* 1999). Digging is suppressed when the sand above them is warmer than their present position (Miller 1996);

- Hatchlings emerge after a rapid decrease in sand temperature within the nest column (Witherington *et al.* 1990). Earlier emergence may be stimulated by an extremely rapid drop in temperature (Hays *et al.* 1992).

Moran *et al.* (1999) concluded that hatchlings respond to a combination of the first two stimuli, although a critical threshold temperature was the most probable controlling factor by thermal inhibition of co-ordinated muscle movement above this temperature. The rate of temperature decline did not appear to play a role in hatchling emergence from natural nests.

There appear to be several advantages of nocturnal emergence: it protects against hyperthermic mortality as hatchlings are unable to regulate their body temperature (Moran *et al.* 1999); lowered activity levels at higher temperatures render hatchlings more vulnerable to predators during the day (Mrosovsky 1968), and the frequency of such predators is less at night (Mrosovsky 1968) (but the diel distribution of aquatic predators on reefs is disputed by Gyuris 1994).

Delay at the sand surface may allow a reduction of blood and muscle lactate levels that have been elevated by anaerobic metabolism during digging. Hatchlings are then capable of the rapid and prolonged flipper-muscle contraction (Dial 1987) required for the crawl down the beach and initial hyperactive swimming activity (see Dial 1987, Wyneken and Salmon 1992). The frenzied rush down the beach is also fuelled by anaerobiosis (Dial 1987), so it is plausible that elevated lactate may be

responsible for the periodic pauses shown by some hatchlings on the beach, although these are quickly broken on physical contact by a sibling whereupon the frenzy continues.

1.2.13 Hatchling Phenotype and Fitness

Teratogenic effects of sub-optimal thermal and hydric conditions during incubation need not be lethal, but may result in abnormal scutellation, and possibly kyphosis and scoliosis (Lynn and Ullrich 1950). Adult turtles with kyphosis and anomalous scales have been observed in the wild (pers.ob.).

The turtle carapace is a composite of the endochondral axial skeleton (the thoracic vertebrae and ribs) and a specialised epidermis overlapping and surrounding the skeletal elements (Burke 1991). The turtle ribs expand and fuse (Hoffstetter and Gasc 1969) to form a rigid box surrounding the limb girdles (shoulder and pelvis) (Zangerl 1969), and elements of the axial skeleton then exert a morphogenetic influence upon the development and position of the dermal elements. Except for the adult flatback turtle (whose epidermis is only thinly keratinised - Limpus *et al.* 1988) and leatherback (whose epidermal shields have been completely reduced), the dermal, bony armour is covered with a hornified epidermal surface. Both the dermal and epidermal components are subdivided into mosaics of discrete geometric shapes, although the patterns on the epidermis (scutes or scales) and underlying dermis are not identical (Zangerl 1969).

The plastral elements are also related to the skeleton (epiplastra with the clavicles, entoplastron with the interclavicle, and remaining plastral plates with the

gastralia or abdominal ribs). Scute anomalies of the carapace and plastron are, therefore, expected to reflect teratogenetic influences (Zangerl 1969) since differentiation of the epidermis occurs well after morphogenesis of the skeleton (Burke 1991). Forsman *et al.* (1994) demonstrated only very weak association between anomalies among captive parents and offspring of the European adder, *Vipera berus*, indicating low heritability of skeletal defects, thereby implicating sub-optimal incubation conditions. While some of the skeletal defects of *V. berus*, such as merged or duplicated ribs, had a direct bearing on locomotory ability, no such demonstration has been made for sea turtles. However, topical scale abnormalities, reflecting as they do underlying imperfections of skeletal element development, may impinge on the physical robustness of hatchlings.

CHAPTER 2

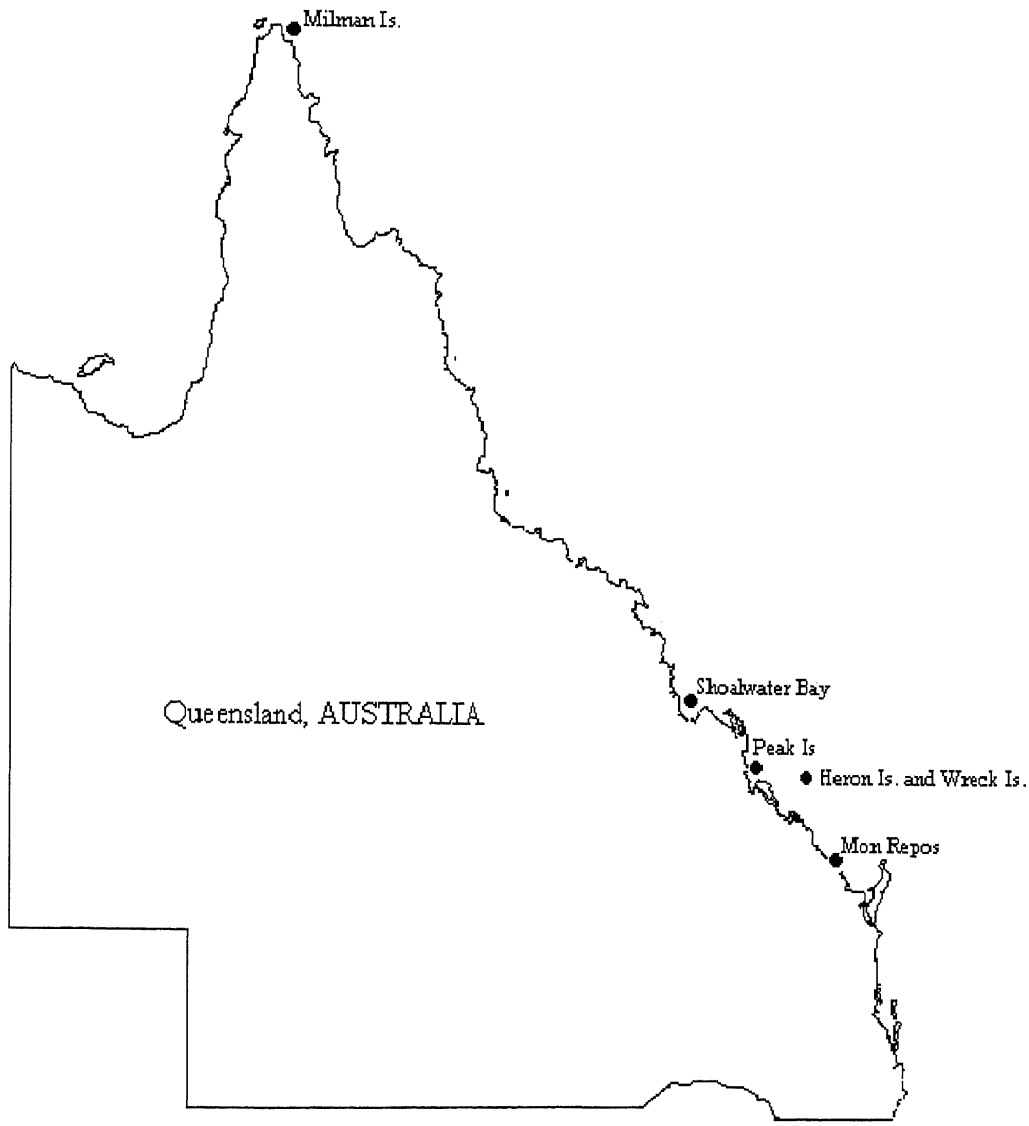
STUDY SITES AND GENERAL METHODS

This study of fungi colonising sea turtle nests in eastern Australia encompassed all the major rookeries and feeding grounds used by the 4 species of turtle that regularly nest in Queensland: the green, loggerhead, flatback and hawksbill. Turtles nesting and/or feeding at Heron Is. and Reef, Milman Is., Mon Repos, Peak Is., Shoalwater Bay and Wreck Is. (see Table 2.1 and Figure 2.1) were investigated to identity fungi appearing on failed eggs, their source(s), methods of egg invasion, and influence upon hatch success. These investigations included the approach or capture of animals, taking of cloacal swabs, nest excavations, culture of fungi, and artificial incubation of eggs for manipulative experimentation.

Table 2.1 Study sites for investigations of fungal colonisation of sea turtle nests in eastern Australia.

Study Site		Turtles Present (by frequency of occurrence)	Turtles Sampled	Year
Foraging Sites	Heron Reef	green, loggerhead, hawksbill	green, loggerhead	1996-1997
	Shoalwater Bay	green, loggerhead, hawksbill, flatback	green	1997
	Heron Is.	green, loggerhead	green, loggerhead	1995-1999
Nesting Sites	Milman Is.	hawksbill, green, flatback	hawksbill, green	1998
	Mon Repos	loggerhead, flatback, green	loggerhead	1998
	Peak Is.	flatback, loggerhead	flatback	1998
	Wreck Is.	green, loggerhead	green, loggerhead	1998

Figure 2.1 Field Sites for the Study of Fungal Colonisation of Sea Turtle Nests in Eastern Australia.



2.1 STUDY SITES

2.1.1 Heron Island

Heron Is. (23°26'S, 151°55'E) is a 16ha coral cay in the Capricorn Group of islands, approximately 70km offshore of Rockhampton. Beach rock comprises 38% of the island's perimeter and varies in width from 9-21m wide on the southern side to 3-6m wide on the northern and western sides. Depending on the extent of sand erosion and deposition, outcropping beach rock may also be present on the eastern side. Although it is only a minor rookery in the Capricorn/Bunker Group, Heron Is. is a principal reference site with long-term baseline data for green turtle studies due to the logistical ease of establishing monitoring groups.

The island is dominated by a central *Pisonia grandis* forest and numerous stands of *Ficus*, *Celtis* or *Pandanus* spp.. Exposed fringing vegetation of *Argusia, Casuarina*, *Cordia*, *Pandanus* spp. and the occasional stand of stunted *P. grandis* is relatively sparse due to harsh weather conditions (Hill and Rosier 1989). For a comprehensive listing of vascular plant see Fosberg (1961), Gillham (1961) and Rogers and Morrison (1994).

Seventy-five bird species have been identified at Heron Is. (Kikkawa 1970), although many may be occasional or vagrant visitors. Black noddies (*Anous minutus*) and wedge-tailed shearwaters (*Puffinus pacificus*) are by far the most numerous during their summer breeding season (see Section 5.3).

Heron Is. is the only island of the Capricorn/Bunker group with a large number of permanent human residents. The construction of a resort, research station and

National Parks station has resulted in some clearing of the *P. grandis* forest, and walking tracks have been constructed through the island core. A retaining wall along 14% of the island perimeter has been constructed by the resort to stabilise the western corner of the island.

2.1.2 Heron Reef

Heron Reef is a lagoonal, large platform reef of approximately 27 km^2, with Heron Is. at its western edge. It has been divided into a Marine National Park 'A' Zone and Marine National Park 'B' Zone by the Great Barrier Reef Marine Park Authority.

The resident green turtles (up to 1000) feed on benthic algae, principally along the reef fronts (with many moving up onto the reef crest at night) and in the lagoon. The loggerhead turtles (estimated at 250 individuals) prey primarily upon molluscs in the lagoonal and reef flat areas. Hawksbill turtles (approximately 50) feed on encrusting animals (e.g. tunicates, sponges, soft corals and molluscs) and algae on the reefs (Limpus *et al.* 1984c).

2.1.3 Milman Island

Milman Is. (11°10'S, 143°00'E) is about 22.7ha in area and located about 112km south-east of Thursday Is. and 45km north-east of Oxford Ness, Queensland, approximately 28km east of the mainland. The island is a sand cay primarily vegetated by closed forest with open shrubs and grasses along the frontal dune. Beach rock occurs on approximately 33% of the beach circumference at low tide, including a 500m section of rock on the western side exposed even at high tide (Loop

et al. 1995). The island is considered to be one of the most significant hawksbill turtle rookeries in the northern Great Barrier Reef (Limpus and Fleay 1983).

The closed forest occupies approximately half of the island and consists predominantly of *Manilkara kauki*, *Erythrina insularis* and *Terminalia muelleria,* while *Ficus* spp., *Guettarda speciosa*, *Ixora klanderiana* and *Mimusops elengi* also contribute to the canopy. The dense forest understorey contains *Malaisia scandens*, *Pleomele angustifolia*, *Capparis* spp., *Carissa laxiflora*, *Jasminum simplicifolium*, *Diospyros maritima*, *D. compacta* and *Drypetes deplanchei.* Shrublands on the remainder of the island include many of the species found in the closed forest, in addition to *Canthium coprosmoides*, *Eugenia reinwardtiana*, *Exocarpos latifolius*, *Micromelum minutum* and *Pittosporum ferrugineum* (Dobbs *et al.* 1997).

A *Casuarina equisetifolia* woodland at the southern end of the island also contains scattered understorey species. Fringing littoral vegetation is characterised by *Premna serratifolia*, *G. speciosa*, *Pemphis acidula* and large overhanging *Manilkara kauki* (Dobbs *et al.* 1997).

Dobbs *et al.* (1997) identified 81 bird species on Milman Is. during 1985 to 1995. Although some seabirds nested on the island and others were observed in breeding plumage, Milman Is. is not considered a major seabird nesting site. It is, however, a significant rest stop for both terrestrial and seabird migratory species (some of which are only present for a few days), so total numbers and species composition vary seasonally.

2.1.4 Mon Repos

Mon Repos Conservation Park (24°48'E, 152°27'E) is on the Australian mainland 13km east of Bundaberg. The beach is approximately 1.5km long at high tide, with 2 rocky areas toward the centre. The smaller 50m long section is inundated at every high tide and the larger 75m long section is only totally submerged at full spring high tide. A small creek empties into the sea at the southern end of the beach (Limpus 1985). In recent years, Mon Repos has replaced Wreck Is. as the largest loggerhead turtle rookery in the southern Pacific Ocean (C.J. Limpus pers.comm.).

The dune structure at Mon Repos is heavily influenced by weather conditions. The fore-dune has a 1-3m elevation (Limpus 1985), but this can change considerably in a single season with the tidal deposition or erosion of sand. In 1906 the original dune forest was cleared (Nolan 1978 in Limpus 1985). Revegetation efforts have resulted in the current *Casuarina equisetifolia* stands that dominate the rear of the dune, with the fore-dune covered by *Spinifex hirsutus* and *Ipomoea pes-caprae* (Limpus 1985).

2.1.5 Peak Island

Peak Is. (23 °20.5'S, 150°56'E) is an uninhabited rocky continental island of the Great Keppel group in the Mackay/Capricorn Section of the Great Barrier Reef Marine Park. The island, approximately 28ha in area and 111m at the highest point, lies 13km off the Central Queensland coast. No permanent freshwater exists. Peak Island is classified as a Preservation Zone within the Great Barrier Reef Marine Park, with access permissible only by research permit.

There are 3 isolated beaches on Peak Is.. The 500m long nesting beach is on the western side, rising from a deep water approach. A second 80m long sandy beach is submerged at spring high tide with a rocky intertidal approach and is therefore unsuitable for both access and nest incubation. The third 80m long beach is composed of shingle (Limpus *et al.* 1981). There have not been any attempted turtle nestings observed at either of the latter two beaches.

Fauna surveys at Peak Is. have identified 6 terrestrial reptiles and 47 birds (Phillott unpubl.). Dune vegetation of the nesting beach is dominated by 3 large *Pandanus* sp. trees with grasses (including *Spinifex sericeus*, *Melinis repens*, *Themeda triandra*, and *Eragrostis sororia*), vines (*Ipomea pes-caprae* and *Passiflora foetida*) and herbaceous plants (*Salsola kali*, *Tribulus terrestris* and *Crotalaria* sp.) on the fore- and rear-dune. The inland vegetation is dominated by *Eucalyptus* spp..

2.1.6 Wreck Island

Wreck Is. (23°21'S, 151°57'E) is a 14ha coral cay adjacent to Heron Is. in the Capricorn Group. It is classified as a Preservation Zone of the Great Barrier Reef Marine Park with access by research permit only. Wreck Is. is the second largest green turtle rookery in the southern Great Barrier Reef (Limpus *et al.* 1984c) and was also the most significant loggerhead turtle rookery in the southern Pacific area until recent years, when nesting numbers have fallen dramatically (C.J. Limpus pers.comm.).

There is well developed beach rock along the southern and north-western side of the cay. The vegetation is similar to that of Heron Is. except that the *P. grandis*

forest is less developed. Like Heron Is., it is a substantial wedge-tailed shearwater breeding site, but there are no nesting black noddies.

2.1.7 Shoalwater Bay

Shoalwater Bay (22°21'S, 150°30'E) on the mainland has extensive shallow mudflats supporting dense sea grass beds upon which green turtles and dugong feed. "Turtle rodeos" (Limpus 1985- see below) were conducted in the western part of the bay between McDonald and Sabina Points, eastward to include Akens Is.. There has been no published estimate of the number of turtles in this area.

2.2 RESEARCH METHODS

2.2.1 Approach of Nesting Animals

Turtles that had come ashore to nest were observed by standard techniques of the Queensland Turtle Research program (QPWS) developed to minimise disturbance. The rear of the egg chamber was excavated by hand during the latter stages of egg chambering to allow collection of eggs or mucus samples during oviposition. This procedure was carried out in low or nil additional light so the turtle was unaware of the observer's presence. Turtles were uniquely identified by numbered titanium self-piercing tags used by the Queensland Turtle Research program.

2.2.2 Capture of Feeding and Courting Animals

Non-nesting animals were caught during the turtle rodeo, described by Limpus (1985). Typically, two 4.28m catch boats ran transects through water of suitable catch depth (if possible <8m, preferably <3m) until a turtle was sighted. A boat was

then manoeuvred alongside the swimming turtle, allowing a catcher to dive in and seize the turtle by its carapace. The turtle was then turned vertically, directing it to swim to the surface, where a catch boat retrieved both turtle and catcher. Running loops were placed around the base of the fore-flippers and the turtle was lifted across the gunwale into the boat.

2.2.3 Procurement of Cloacal Swabs

TRANSTUBE® Transport Medium (Amies Clear Media), manufactured by the Medical Wire and Equipment Co. Ltd., Corsham, Wiltshire, England (Code MW170), were used to sample the cloacal mycobiota of nesting and non-nesting (feeding or courting) sea turtles. Post-nesting females were inverted as they left the beach and the exterior of the cloaca irrigated with sterile, distilled water. Animals captured during the turtle rodeo were balanced on the edge of the gunwale as they were lifted aboard the boat. The swabs were inserted 12cm into the cloaca and gently rotated during sampling. Samples were stored at 3-5^{0}C until plating for subsequent identification of mycobiota.

2.2.4 Collection of Eggs

Eggs were taken for anatomical, anti-microbial property and incubation studies. Single-use gloves were worn during all egg handling procedures. Multiple-gloving prior to the commencement of oviposition allowed contaminated gloves to be quickly discarded, thus avoiding any loss of eggs.

Eggs were placed directly into Sarstedt® autoclave disposal bags, that were supported in a 10L plastic bucket to help hold the weight of the eggs and prevent

weakening and tearing of the bag under stress. The neck of each bag was twisted and folded over before securing with a rubber band. The exterior of each bag was labelled with permanent marker.

2.2.5 Transport of Eggs

Eggs were transported to the laboratory following the procedures of Harry and Limpus (1989) for long distance transport. Eggs were chilled to 7-10°C (within 2hrs of oviposition) and held for no longer than 48hrs, allowing collection of multiple clutches over successive nights and subsequent transport. Collection bags were not opened during temperature depression to minimise exposure to microbes. Bags were arranged in large insulated foam containers so they were stable, and air spaces between them filled with clean, expanded polystyrene pellets for support and insulation.

2.2.6 Artificial Incubation of Eggs

Prior to egg collection, incubators were cleaned with a 5% sodium hypochlorite bleach solution and then rinsed with a 5% solution of disinfectant. At the same time, incubation containers (for holding the eggs) were sterilised either by autoclaving at 121°C for 15mins with the mouth covered in aluminium foil or washing with a 5% bleach solution followed by a sterile water rinse. As incubation container size differed among experiments, these details are given in the relevant chapters and sections.

Sand to be used as an incubation substrate was collected from areas of the nesting beach relatively free of organic material. It was sterilised by autoclaving in

small lots, with thermal sentinels (e.g. Thermalog®S strips) placed within samples to check that heat had penetrated to the core.

The exterior of each incubation container was uniquely labelled and individual eggs annotated with graphite pencil; no biodegradable labels were placed inside the container. The required moisture conditions were maintained by adding sterile water either from clean spray bottles, or by use of a sub-surface trickle irrigator.

2.2.7 Handling of Eggs

As described above, single-use sterile gloves were worn and eggs handled in a room or area with minimal air flow or disturbance. To minimise movement induced mortality, egg orientation was maintained (Limpus *et al.* 1979, Parmenter 1980).

Prior to each session of use, equipment was cleaned with a 5% bleach solution and rinsed with sterile distilled water. Any sand that had to be removed from the egg was done so with a soft brush that would not disrupt the structural integrity of the eggshell which could potentially allow pathogens access to the egg contents.

2.2.8 Excavation of Emerged Nests

Emerged nests were detected by the presence of characteristic sinkholes that formed with the reduction of nest volume at hatchling emergence, the presence of hatchling tracks, or by observing hatchlings emerging from the nest. The contents of the egg chamber were removed by hand and sorted into the following categories:

Shell eggshell, greater than half the size of an egg at oviposition;

Live Hatchlings live hatchlings remaining in the base of the egg chamber, which would not have been able to emerge without human intervention;

Dead Hatchlings carcasses in the neck or base of the egg chamber;

Undeveloped Eggs entire eggs whose contents did not demonstrate any visible signs of embryonic development (such as a blood spot);

Unhatched Eggs eggs containing embryos at various stages of development, including fully developed embyos that died prior to complete emergence from the shell;

Predated Eggs entire eggs without their egg contents and a small perforation (usually the only indication of predation).

Counts of all eggs/eggshell meeting the above criteria were recorded on a standard proforma and used to calculate the following:

Clutch Count = Eggshell + Undeveloped + Unhatched + Predated

% Hatch Success = (Eggshell/Clutch Count) x 100

% Emergence Success = ((Eggshell-Live Hatchlings-Dead Hatchlings)/ Clutch Count) x 100

Separate counts were kept of 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 = (Undeveloped with Fungi + Unhatched with Fungi)/(Undeveloped + Unhatched) x 100

% Clutch with Fungi = (Undeveloped with Fungi + Unhatched with Fungi)/ Clutch Count x 100

These nest descriptors were used in determining differences in productivity and fungal colonisation of nests between turtle species, rookery, and nest habitat.

This study was carried out with Central Queensland University Animal Experimentation Ethics Panel and Queensland Parks and Wildlife Service approval.

CHAPTER 3

VARIATION IN HATCH SUCCESS AND OCCURRENCE OF FUNGI IN SEA TURTLE NESTS IN EASTERN AUSTRALIA

3.1 INTRODUCTION

The hatch success of sea turtle nests is calculated as the percentage of eggs producing hatchlings that fully emerge from the eggshell. Mortality during the early stages of sea turtle embryonic development is relatively high (Richardson and Richardson 1982), and excavations of emerged nests have indicated that a significant percentage of eggs fail to hatch (Bustard 1972, Fowler 1979, Whitmore and Dutton 1985). This is usually attributed to erosion, depredation, plant root invasion, excessive rainfall, tidal inundation and developmental abnormalities. Movement-induced mortality may be an additional cause of mortality in relocated (i.e. artificial) nests (Limpus *et al.* 1979, Limpus 1980c, Parmenter 1980, Blanck and Sawyer 1981, Whitmore and Dutton 1985). Some eggs are classified as infertile (Bustard 1972, Fowler 1979, Stancyk *et al.* 1980, Whitmore and Dutton 1985), but since most unhatched eggs are not inspected until after nest emergence, early embryonic death and subsequent decomposition during the intervening time may be inappropriately interpreted as "infertility" (Parmenter 1980, Wyneken *et al.* 1988).

Limpus *et al.* (1983d) considered the lower hatch success of loggerhead nests at Heron Is. relative to Mon Repos (see Table 1.2.4) could be related to the presence of fungi on failed eggs. The possible association of fungi with egg failure has not been considered at other eastern Australia nesting beaches. To investigate the potential role of fungi in loggerhead nest failure at Heron Is., the hatch success and

percentage of eggs colonised by fungi at this site were compared with other rookeries.

3.2 PROCEDURE

Emerged nests at Heron, Milman, Peak and Wreck Islands and Mon Repos were located and excavated as described in Chapter 2. The hatch and emergence success was calculated for each nest (see Chapter 2) and used to determine the mean hatch and emergence success for each rookery. The relationship between hatch and emergence success for all nests was determined by correlation and matched-pair t-tests. Variation in hatch success among turtle species and rookeries was analysed using one-way analyses of variance with *post-hoc* Tukey tests where appropriate.

The percentage of failed eggs and percentage of the clutch with fungi was also calculated for each nest (see Chapter 2). Variation in fungal colonisation of failed eggs and of the entire clutch among turtle species and rookeries was analysed using one-way analyses of variance with *post-hoc* Tukey tests where appropriate.

All percentile data met the criteria of normal distribution and did not require transformation prior to statistical analyses (see Zar 1999).

3.3 RESULTS

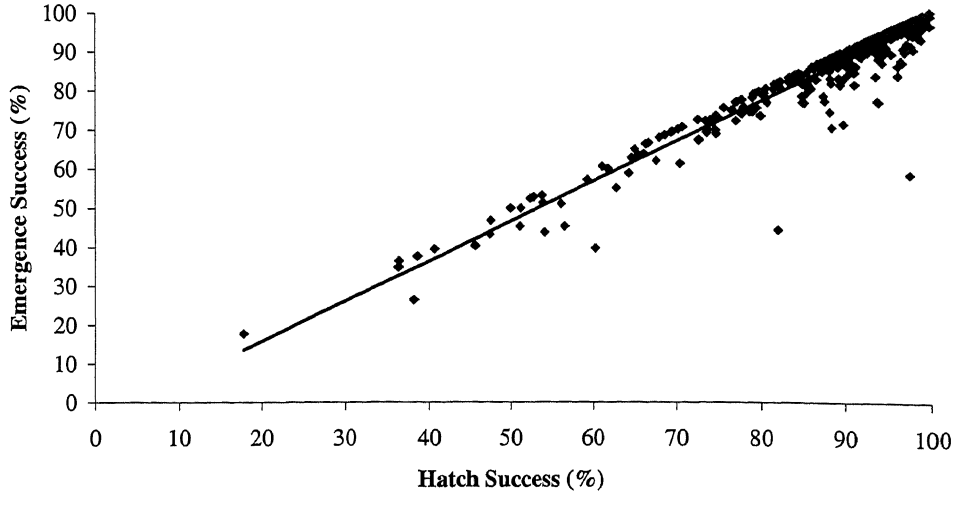
The hatch and emergence success for sea turtles nesting in eastern Australian is summarised in Table 3.1. Obviously, emergence success can never be greater than hatch success, but a matched-pair t-test found emergence success to be significantly less than hatch success (t_{341}=10.840, P<0.001) for all nests of all species at all

rookeries. There was also a significant correlation (r=0.954, $P<0.001$) between these two measures of nest productivity (see Figure 3.1) so further statistical analyses were carried out on hatch success alone. Sampling year (1996 and 1997) had no significant effect on the hatch success of green or loggerhead turtle nests at Heron Is. ($F_{1, 197}=0.016$, $P=0.899$), allowing data for each species at this site to be pooled.

Table 3.1 The hatch success of sea turtles nests at Heron Is., Milman Is., Mon Repos, Peak Is. and Wreck Is.. n indicates the number of clutches examined.

Rookery (Year), Turtle	n	Hatch Success % Mean±SD (range)	Emergence Success % Mean±SD (range)
Heron Island (1996), Green	86	88.7±11.0 (47.4-100.0)	87.0±12.00 (43.3-70.3)
Heron Island (1996), Loggerhead	2	65.7 (61.2-70.3)	65.4 (60.6-70.3)
Heron Island (1997), Green	108	89.4±10.7 (65.0-100.0)	85.8±10.1 (65.0-100.0)
Heron Island (1997), Loggerhead	5	63.7±8.9 (56.1-77.8)	60.1±12.8 (45.3-77.8)
Milman Island (1998), Green	7	88.1±7.5 (77.2-97.0)	87.6±7.0 (77.2-96.0)
Milman Island (1998), Hawksbill	32	87.8±7.5 (77.2-99.2)	87.5±7.0 (77.2-99.2)
Mon Repos (1998), Loggerhead	12	90.5±5.2 (83.5-99.2)	87.9±5.2 (83.2-96.6)
Peak Island (1998), Flatback	44	81.2±17.7 (17.8-100.0)	78.3±18.3 (17.8-100.0)
Wreck Island (1998), Green	39	88.6±10.1 (51.2-99.1)	86.1±10.6 (50.0-99.1)
Wreck Island (1998), Loggerhead	7	73.9±20.2 (38.61-97.12)	67.4±22.4 (37.6-95.2)

Figure 3.1 The Correlation Between Hatch and Emergence Success of Sea Turtle Nests (r=0.954).



The hatch success of loggerhead nests varied significantly among rookeries (analyses of variance statistics in Table 3.2), with significantly greater hatching at Mon Repos than at Heron Is. (Tukey $P<0.001$) and Wreck Is. (Tukey $P=0.017$). There was no significant difference in hatch success of loggerhead nests between Heron Is. and Wreck Is. (Tukey $P=0.287$). The hatch success of green turtle nests was not influenced by rookery (Table 3.2). Hatch success differed significantly among turtle species at Heron Is. and Wreck Is., but not at Milman Is. (Table 3.3).

Table 3.2 Results of one-way analyses of variance for the influence of rookery on intra-specific variation in sea turtle hatch success. Subsets for *C. caretta* are significantly different, as determined by *a posteriori* Tukey tests.

	Rookery			
Turtle	Mon Repos	Heron Island	Wreck Island	Milman Island
Loggerhead	$F_{2, 23}=12.088$, $P<0.001$			-
	Subset 1	Subset 2		
Green	-	$F_{2, 237}=0.056$, $P=0.946$		

- indicates no data available for particular combination of turtle species and rookery

Table 3.3 Results of one-way analyses of variance for the influence of sea turtle species on intra-rookery variation in hatch success.

	Turtle		
Rookery	Loggerhead	Green	Hawksbill
Heron Island	$F_{1, 199}=31.980$, $P<0.001$		-
Milman Island	-	$F_{1, 37}=0.004$, $P=0.953$	
Wreck Island	$F_{1, 44}=8.917$, $P=0.005$		-

- indicates no data available for particular combination of turtle species and rookery

Fungi were found in sea turtle nests at all rookeries (Table 3.4). The effect on the nest was calculated as both the percentage of failed eggs and percentage of the clutch colonised by fungi and these are discussed, in turn, below.

Table 3.4 The percentage of failed eggs and percentage of the clutch colonised by fungi at Heron Is., Milman Is., Mon Repos, Peak Is. and Wreck Is.. n indicates the number of clutches examined.

Rookery (Year), Species	n	% failed eggs with fungi Mean±SD (range)	% clutch with fungi Mean±SD (range)
Heron Island (1996), Green	86	72.81±38.46 (0.00-100.00)	9.48±11.04 (0.00-52.58)
Heron Island (1996), Loggerhead	2	93.18±9.64 (86.36-100.00)	31.96±8.88 (25.68-38.24)
Heron Island (1997), Green	108	80.59±43.56 (0.00-100.00)	9.23±11.28 (0.00-34.96)
Heron Island (1997), Loggerhead	5	96.59±7.64 (82.93-100.00)	33.69±7.54 (22.22-43.40)
Milman Island (1998), Green	7	85.71±37.80 (0.00-100.00)	10.17±6.72 (0.00-17.72)
Milman Island (1998), Hawksbill	32	84.39±37.80 (0.00-100.00)	10.42±6.72 (0.00-63.57)
Mon Repos (1998), Loggerhead	12	27.13±39.62 (0.00-100.00)	3.26±5.85 (0.00-15.86)
Peak Island (1998), Flatback	44	77.64±34.94 (0.00-100.00)	15.19±16.96 (0.00-82.22)
Wreck Island (1998), Green	39	89.15±24.73 (0.00-100.00)	10.38±9.92 (0.00-46.23)
Wreck Island (1998), Loggerhead	7	100.00±0.00	25.01±19.75 (2.88-60.40)

For failed eggs colonised by fungi, there was a significant difference amongst rookeries for loggerhead eggs (Table 3.5), with significantly fewer failed eggs colonised at Mon Repos than at Heron Is. (Tukey $P<0.001$) or Wreck Is. (Tukey $P<0.001$) but no significant difference between Heron Is. and Wreck Is. (Tukey $P=0.953$). In contrast, the colonisation of failed green turtle eggs was not significantly different among rookeries (Table 3.5). The percentage of failed eggs affected by fungi did not differ significantly among turtle species at Heron Is., Wreck Is. or Milman Is. (Table 3.6).

Table 3.5 Results of one-way analyses of variance for the influence of rookery on intra-specific variation in the percentage of failed eggs colonised by fungi. Subsets for *C. caretta* are significantly different, as determined by *a posteriori* Tukey tests.

	Rookery			
Turtle	Mon Repos	Heron Island	Wreck Island	Milman Island
Loggerhead	$F_{2, 23}$=21.126, P<0.001			-
	Subset 1	Subset 2		
Green	-	$F_{2, 237}$=1.995, P=0.138		

- indicates no data available for particular combination of turtle species and rookery

Table 3.6 Results of one-way analyses of variance for the influence of sea turtle species on intra-rookery variation in the percentage of failed eggs colonised by fungi.

	Turtle		
Rookery	Loggerhead	Green	Hawksbill
Heron Island	$F_{1, 199}$=1.737, P=0.189		-
Milman Island	-	$F_{1, 37}$=0.008, P=0.929	
Wreck Island	$F_{1, 44}$=1.323, P=0.256		-

- indicates no data available for particular combination of turtle species and rookery

The percentage of the total loggerhead clutch affected by fungi differed significantly among rookeries. A significantly smaller percentage of each clutch was affected at Mon Repos than at Heron Is. (Tukey P<0.001) and Wreck Is. (Tukey P=0.002) with no difference between Heron Is. and Wreck Is. (Tukey P=0.391). The percentage of the green turtle clutch affected by fungi was not influenced by rookery (Table 3.7). The percentage of the total clutch affected by fungi was significantly different between turtle species at Heron Is. and Wreck Is., but not at Milman Is. (Table 3.8).

Table 3.7 Results of one-way analyses of variance for the influence of rookery on intra-specific variation in the percentage of the total clutch colonised by fungi. Subsets for *C. caretta* are significantly different, as determined by *a posteriori* Tukey tests.

	Rookery			
Turtle	Mon Repos	Heron Island	Wreck Island	Milman Island
Loggerhead	$F_{2, 23}$=17.278, P<0.001			-
	Subset 1	Subset 2		
Green	-	$F_{2, 237}$=0.152, P=0.859		

- indicates no data available for particular combination of turtle species and rookery

Table 3.8 Results of one-way analyses of variance for the influence of sea turtle species on intra-rookery variation in the percentage of the total clutch colonised by fungi.

	Turtle		
Rookery	Loggerhead	Green	Hawksbill
Heron Island	$F_{1, 199}$=29.558, P<0.001		-
Milman Island	-	$F_{1, 37}$=0.002, df=1, P=0.967	
Wreck Island	$F_{1, 44}$=9.200, P=0.004		-

- indicates no data available for particular combination of turtle species and rookery

3.4 DISCUSSION

The hatch success of loggerhead nests at Heron Is. and Wreck Is. was significantly less than at Mon Repos. In contrast, green turtles nesting at Heron Is. and Wreck Is. had a similar hatch success to that of the other rookery (Milman Island) and of other species elsewhere (loggerhead turtles at Mon Repos, hawksbill turtles at Milman Is. and flatback turtles at Peak Is.). This suggests some characteristic of Heron Is. and Wreck Is. nesting beaches is not optimal for loggerhead turtle nests.

Failed loggerhead eggs at Heron Is. and Wreck Is. were more likely to be invaded by fungi than at Mon Repos. At these islands, the percentage of the total clutch (but not the percentage of the failed eggs) colonised by fungi also differed significantly between turtle species. This was not observed at Milman Is. where

there was no significant difference in either the percentage of failed green or hawksbill turtle eggs or percentage of the green or hawksbill turtle clutch affected by fungi.

Therefore, inter-specific variation in both hatch success and the fungal colonisation of sea turtle nests is restricted to Heron Is. and Wreck Is.. Loggerhead nests at Heron Is. and Wreck Is. have a lower hatch success and higher percentage of the clutch affected by fungi. As green turtle nests at the same islands have a similar colonisation rate of failed eggs, but a significantly higher hatch success and lower percentage of the clutch demonstrating fungal growth, it is possible that fungi may be contributing to the high loggerhead embryo mortality observed at Heron Is. and Wreck Is.. The fungi isolated from failed sea turtle eggs are discussed in Chapter 4.

CHAPTER 4

FUNGI IDENTIFIED FROM SEA TURTLE NESTS IN EASTERN AUSTRALIA

4.1 INTRODUCTION

Fungi have been isolated from the egg exterior and/or embryonic tissue of several species of sea turtle including the loggerhead (Ragotzkie 1959, Peters *et al.* 1994, Wyneken *et al.* 1988), green (Bustard and Greenham 1968, Solomon and Baird 1980, Whitmore and Dutton 1985), leatherback (Whitmore and Dutton 1985, Chan and Solomon 1989, Eckert and Eckert 1990) and olive ridley (Mo *et al.* 1990, Acuña-Mesén 1992) turtles. Most observations are of "black" eggs, or discoloured egg contents and embryonic material (Table 4.1). There have been no published descriptions of the nest mycobiota, or its potential contribution to egg failure, at eastern Australia rookeries. The high failure of loggerhead nests at Heron and Wreck Is. and the high percentage of the clutch colonised by fungi (see Chapter 3) suggested mycobiota may be contributing to loggerhead embryo mortality at these rookeries.

4.2 PROCEDURE

During the assessment of hatch success in emerged nests at eastern Australia rookeries, a single egg that appeared contaminated (i.e. the shell was blackened with fungi) from each nest was swabbed. Fungal swabs were incubated on half-strength Potato Dextrose Agar with 0.05g L^{-1} chloramphenicol to inhibit bacterial growth and then subcultured onto a range of media (e.g. Potato Dextrose Agar, Nutrient Agar, Carnation Leaf Agar) as required for identification. Identification of species not

Table 4.1 Descriptions of fungi on sea turtle eggs.

Turtle	Rookery	Fungi	Observation	Source
Loggerhead	Sapelo Is., Georgia, U.S.A.	–	Strong H_2S odour, black eggs	Ragotzkie 1959
	Göksu Delta, Turkey	*Mucor*	Black contents and/or shell	Peters *et al.* 1994
	Jekyll Is., Georgia, U.S.A.	–	Discolourations	Wyneken *et al.* 1988
Green	Heron Is., Queensland, Australia	–	Loss of turgidity, blackish mould	Bustard and Greenham 1968
	-	*Aspergillus*	–	Solomon and Baird 1980
	Suriname	–	Decomposed egg contents, discoloured shell	Whitmore and Dutton 1985
Leatherback	Suriname	–	Decomposed egg contents, discoloured shell	Whitmore and Dutton 1985
	Rantau Abang, Malaysia	–	Rust or grey discolouration of shell	Chan and Solomon 1989
	St Croix, U.S. Virgin Islands	*Fusarium*	Embryos heavily infested with fungi and/or bacteria	Eckert and Eckert 1990
Olive Ridley	Nancite, Costa Rica	*Allescheria, Aspergillus, Cunninghamella, Gliocladiopsis, Fusarium, Homodendrum, Saksenaea*	–	Mo *et al.* 1990
	Naranjo and Nancite, Costa Rica	*Fusarium, Monosporium*	–	Acuña-Mesén 1992

– indicates no data available

included by Booth (1971) and Seifert (1996) was based on previous unpublished work (C.J. Limpus pers.comm.) and confirmed by Prof. D.E. Ellis from the Adelaide Women's and Children's Hospital.

The influence of fungal species on the hatch success, percentage of failed eggs colonised by fungi, and percentage of the total clutch colonised by fungi (as determined in Chapter 3) was analysed for each species nesting at each rookery by one-way analyses of variance. Nests that had been disturbed by other turtles or inundated by the high tide were excluded from these analyses. Analyses of variance were not carried out on data sets where sample sizes were too small.

4.3 RESULTS

Three species of fungi (*Fusarium oxysporum*, *Fusarium solani* and *Pseudallescheria boydii*) were frequently isolated from failed eggs in sea turtle nests (see Table 4.2). Most samples were of single species, but 6.54% of all swabs revealed mixed cultures (14.29% *F. oxysporum* + *F. solani*; 28.57% *F. oxysporum* + *P. boydii*; 57.14% *F. solani* + *P. boydii*) (see Table 4.2 where totals exceed 100% for some samples). The percentage of nests containing each fungal species varied among rookeries and also between nesting seasons at Heron Is. (the only rookery to be sampled in more than one year).

There was no significant difference among fungus species on the hatch success (Table 4.3), the percentage of failed eggs colonised by fungi (Table 4.4) or the percentage of the total clutch colonised by fungi (Table 4.5) at any rookery. Data for

hatch success and fungal colonisation of eggs has been pooled for each fungal species in Table 4.6.

Table 4.2 Spatial and temporal variation in fungal presence in sea turtle nests at Heron Is., Milman Is., Mon Repos, Peak Is. and Wreck Is.. Row totals exceed 100% in some cases because of multiple isolates from some samples.

			% Nests		
Rookery	Turtle	n	*F. oxysporum*	*F. solani*	*P. boydii*
Heron Island 1996/97	Green	22	22.7	63.6	13.6
	Loggerhead	8	0.0	100.0	0.0
Heron Island 1997/98	Green	20	5.0	5.0	100.0
	Loggerhead	3	0.0	0.0	100.0
Milman Island	Green	3	33.0	66.0	0.0
	Hawksbill	8	12.5	75.0	12.5
Mon Repos	Loggerhead	5	20.0	100.0	20.0
Peak Island	Flatback	16	12.5	62.5	25.0
Wreck Island	Green	16	18.8	62.5	37.5
	Loggerhead	4	50.0	50.0	25.0

Table 4.3 Results of one-way analyses of variance for the influence of *F. oxysporum*, *F. solani* and *P. boydii* on the hatch success of sea turtle nests.

		Fungi		
Rookery	Turtle	*F. oxysporum*	*F. solani*	*P. boydii*
Heron Is.	Loggerhead	–	$F_{1,3}=0.700$, $P>0.05$	
	Green		$F_{2,25}=0.621$, $P>0.05$	
Milman Is.	Green		ANOVA not possible	–
	Hawksbill		$F_{2,5}=0.765$, $P>0.05$	
Mon Repos	Loggerhead		$F_{2,3}=0.077$, $P>0.05$	
Peak Is.	Flatback		$F_{2,9}=0.917$, $P>0.05$	
Wreck Is.	Loggerhead		$F_{2,2}=3.912$, $P>0.05$	
	Green		$F_{2,15}=0.196$, $P>0.05$	

– indicates nil detection of that fungal species

Table 4.4 Results of one-way analyses of variance for the influence of *F. oxysporum*, *F. solani* and *P. boydii* on the percentage of failed sea turtle eggs colonised by fungi.

		Fungi		
Rookery	Turtle	*F. oxysporum*	*F. solani*	*P. boydii*
Heron Is.	Loggerhead	–	$F_{1,3}$=0.016, P>0.05	
	Green		$F_{2,25}$=0.346, P>0.05	
Milman Is.	Green	% Failed Eggs Colonised by Fungi=100%,n=3		–
	Hawksbill		$F_{2,5}$=0.125, P>0.05	
Mon Repos	Loggerhead		$F_{2,3}$=0.745, P>0.05	
Peak Is.	Flatback		$F_{2,9}$=0.467, P>0.05	
Wreck Is.	Loggerhead		% Failed Eggs Colonised by Fungi=100%, n=5	
	Green		$F_{2,15}$=0.645, P>0.05	

– indicates nil detection of fungal species

Table 4.5 Results of one-way analyses of variance for the influence of *F. oxysporum*, *F. solani* and *P. boydii* on the percentage of the sea turtle clutch colonised by fungi.

		Fungi		
Rookery	Turtle	*F. oxysporum*	*F. solani*	*P. boydii*
Heron Is.	Loggerhead	-	$F_{1,3}$=0.244, P>0.05	
	Green		$F_{2,25}$=0.539, P>0.05	
Milman Is.	Green		ANOVA not possible	-
	Hawksbill		$F_{2,5}$=0.329, P>0.05	
Mon Repos	Loggerhead		$F_{2,3}$=0.716, P>0.05	
Peak Is.	Flatback		$F_{2,9}$=0.909, P>0.05	
Wreck Is.	Loggerhead		$F_{2,2}$=3.796, P>0.05	
	Green		$F_{2,15}$=0.271, P>0.05	

– indicates nil detection of fungal species

Table 4.6 The influence of *F. oxypsorum*, *F. solani* and *P. boydii* on accumulated hatch success and fungal colonisation of sea turtle nests in eastern Australia. Mean±SD (Range).

Fungus	Number of Nests	% Hatch Success	% Failed Eggs Invaded by Fungus	% Clutch Invaded by Fungus
F. oxysporum	14	82.8±20.4 (17.8-97.1)	84.4±31.1 (8.3-100.0)	21.6±30.9 (1.2-100.0)
F. solani	37	76.7±15.5 (36.4-95.1)	84.5±30.9 (7.7-100.0)	20.5±16.7 (0.8-62.1)
P. boydii	34	79.3±17.6 (40.7-99.0)	94.3±17.6 (14.3-100.0)	19.6±17.1 (0.9-58.2)

4.4 DISCUSSION

F. oxysporum and *F. solani* are common, cosmopolitan soil saprophytes (Burgess 1981) that have also been isolated from littoral habitats (see review by Stoner 1981). *F. solani* has been implicated in the poor health of loggerhead hatchlings emerging from heavily infested soil (Rebell *et al.* 1971), and unidentified *Fusarium* spp. have been isolated from the eggshells of unhatched olive ridley turtle eggs in Costa Rica (Mo *et al.* 1990, Acuña-Mesén 1992). *F. solani* is also an infectant of eggs and embryos of the salt-water crocodile (*Crocodylus porosus*) (Hibberd and Harrower 1993), while eggs of the American alligator (*Alligator mississippiensis*) appear to be susceptible to infection by *F. oxysporum* (Schumacher and Cardeilhac 1990).

P. boydii is commonly found in soil and water and has a circumglobal distribution (Rippon 1982) including marine soils (Dabrowa *et al.* 1964, Kirk 1967). This fungus does not have a high inherent virulence, but is described in veterinary and human medicine as an opportunistic infectant (Rippon 1988). It has previously been isolated from olive ridley nests in Costa Rica as *Allescheria* sp. (Mo *et al.* 1990) and *Monosporium apiospermum*, the anamorph of *P. boydii* (Acuña-Mesén 1992).

The contamination of non-viable eggs by mycobiota naturally occurring within the nest substrate is not surprising, but it is also possible that the fungi are acting as pathogens. Solomon and Tippett (1987) and Wyneken *et al.* (1988) describe the failure of eggs in clusters within natural and artificial nests, thus suggesting an infectious aetiology. The role of fungi as infectant or opportunistic contaminant is examined later in this study. There was no significant difference among *F. solani*,

F. oxysporum or *P. boydii* on hatch success or invasion of eggs of any turtle species at any rookery. On this basis, there are no grounds for categorising any of the fungal species as either infectants or contaminants. The greater mortality of loggerhead eggs at Heron Is. and Wreck Is. (Chapter 3) may be due to either a lower resistance of viable eggs to mycobiotic invasion, or fatality due to other influences leading to successive fungal colonisation.

The temporal variation in predominant fungi observed at Heron Is. (1996 - *F. solani*; 1997 - *P. boydii*) suggests that spatial differences in species composition may not necessarily be due to geographic location and instead may be caused by variation in thermal, hydric and/or physical properties of the substrate. A comparison of rainfall (1996- 237mm; 1997- 183mm) and air temperature (daily $\bar{X}\pm SD$ 1996- max. 29.8±1.7°C, min. 23.1±1.6°C; 1997- max. 30.9±2.0°C, min. 24.0±1.9°C; beach temperature data were not available) by paired samples t-test showed average daily temperature differed significantly (t_{238}= 232.443, P<0.001) at Heron Is. over the November to February sampling period between the 2 years. There was no significant difference in daily rainfall between the 2 years (t_{239}= 0.405, P=0.405). Variation in temperature may have contributed to the change in dominant nest mycobiota, as *F. solani* is more prevalent in areas with relatively low temperature (Burgess and Summerell 1992). The germination rate and hyphal growth of the 3 fungi at hydric and thermal micro-climatic conditions experienced within natural sea turtle nests is examined in Chapter 9.

CHAPTER 5

THE SOURCE OF FUNGI INVADING SEA TURTLE EGGS

Since *F. oxysporum*, *F. solani* and *P. boydii* are common soil saprophytes, their most likely source is the nest substrate. However, Wyneken *et al.* (1988) attributed low hatch success in loggerhead turtles to pathogens transferred from turtle to egg via intra-oviductal infection. This may account for some egg mortality at all rookeries, but does not explain the higher mortality of loggerhead eggs at Heron Is. and Wreck Is.. Two obvious features distinguish Heron Is. and Wreck Is. from other rookeries: the type of vegetation and the population density of seabirds. Consequently, all of these factors were investigated to determine whether they contributed to fungal invasion of turtle eggs.

5.1 THE POTENTIAL FOR INTRA-OVIDUCTAL FUNGAL CONTAMINATION OF SEA TURTLE EGGS

5.1.1 INTRODUCTION

Loggerhead, green and flatback turtles often evert their ovipositor into contact with the beach sand during the construction of body pits and egg chambers (Phillott pers.ob.). Eversion and subsequent exposure to the nest substrate during laying allows sand to accumulate on the exterior of the ovipositor. Upon retraction of the ovipositor into the cloaca, soil microbiota can be drawn into the reproductive tract.

The prolonged survival and viability of intra-oviductal spermatozoa suggests that any biocontaminants accumulated during the nesting process may also remain

viable for an extended period of time. A single mating period occurs up to 2 months before the first oviposition of the nesting season (Frazier 1971, Booth and Peters 1972, Limpus and Miller 1993). There is no indication of decreased fertility in clutches laid throughout a season (Limpus 1985), so conditions within the oviduct must be conducive to spermatozoan survival. Although the availability of breeding males at courtship areas (Limpus 1993) gives little selective advantage to fertilisation using spermatozoa from previous breeding seasons, reptilian spermatozoa may remain viable within storage areas for up to 7 years (Birkhead and Møller 1993).

Oviductal immuno-suppression of spermatozoa appears very weak in sea turtles as several hybrids have been recorded: green × hawksbill (Wood *et al.* 1983), loggerhead × hawksbill (Kamezaki 1983), green × loggerhead (C.J. Limpus pers.comm.) and loggerhead × Kemp's ridley (Karl *et al.* 1995). It is estimated the 2 tribes within the Cheloniidae, Chelonini (*Chelonia*) and Caretini (*Caretta, Eretmochelys* and *Lepidochelys*), have been separated for 50-70 million years (Bowen *et al.* 1993, Ernst and Barbour 1989, although disputed by Zangerl 1980). Lineages within the Caretini probably separated 10-20 million years ago (Dodd and Morgan 1992, Zangerl 1980). The successful production of hybrids is, therefore, surprising as species-specific barriers to sperm viability do occur in similarly divergent bird species (Steele and Wishart 1992).

Both the production of hybrid offspring and successful long-term storage of sea turtle spermatozoa suggests oviductal physiological conditions (oxygen tension, ionic and organic components of luminal fluids, and pH) and immune responses are favourable for the extended survival of cells, the equivalent of which, in other

animals, may be capable of eliciting a phagocytic attack by the host. Therefore, oviductal conditions may also allow the survival of fungal spores accumulated during nesting.

5.1.2 PROCEDURE

To determine the presence and possible longevity of fungal spores in the lower oviduct, Transtube® (Amies Clear Media) swabs were inserted 12cm into the cloaca and samples then refrigerated at 3-5°C prior to analysis. Female turtles were sampled at a number of locations (see Tables 5.1-5.6). Most individuals were sampled immediately after capture by the "rodeo method", and their breeding status and history determined by laparoscopic examination (Limpus and Reed 1985a). Nesting animals were inverted as they prepared to leave the beach at the completion of oviposition, swabbed, and then released. All turtles were identified by their individually numbered tag applied by Queensland Turtle Research, Queensland Parks and Wildlife Service (Limpus and Reed 1985a).

Animals were categorised according to their phase of breeding and potential exposure to soil mycobiota as: pre-gravid animals of adult size that had never nested and were captured feeding; animals that had bred ≥2 years ago and were captured feeding; animals that had bred ≥2 years ago and were captured courting; animals that were captured on the beach after oviposition; animals that were captured in the inter-nesting habitat after oviposition; and animals that had bred in the previous season (≤1 year ago) and were captured feeding.

A single, freshly dead, green turtle at Raine Is. (11°243'S, 144°00'E) was swabbed at the cranial, medial and caudal parts of the right oviduct and at the cloaca. Observation of *corpora albicantia* and *corpora lutea* on the ovaries indicated the animal had nested during a previous breeding season and already laid a clutch in the current season. A lack of oviductal eggs attested to successful oviposition before the turtle had overturned on the steep sand bank, where death due to over-heating had occurred earlier in the same day as sampling.

Swabs were initially plated onto half-strength Potato Dextrose Agar, then sub-cultured and identified according to procedures of Booth (1971), Ellis (1971), Pitt (1979), Carmichael *et al.* (1980), Barnett and Hunter (1986), Klich and Pitt (1988) and Hanlin (1990). The incidence of cloacal mycobiota among nesting and non-nesting turtles was analysed with a Chi-square test for heterogeneity.

5.1.3 RESULTS

A total of 133 turtles were swabbed to determine their cloacal mycobiota (see Tables 5.1.1 - 5.1.6). Pre-gravid (17% of 12) female turtles and mature animals that had not bred for ≥2 years (13% of 30) demonstrated the cloacal presence of *Acremonium*, *Cladosporium* or *Penicillium* as a single isolate for any positive swab. Courting females had higher rates (30% of 27) of *Cladosporium* or *Penicillium* but again as the occurrence of only a single taxon in any given turtle. Nesting (75% of 51) and inter-nesting (100% of 2) turtles presented the highest rates and several genera (*Acremonium*, *Aspergillus*, *Chrysosporium*, *Fusarium*, *Mucor*, *Penicillium*, *Phialophora*, *Sporothrix* and *Stachybotrys*). In addition, several nesting individuals swabbed positive for more than one fungus. Female turtles at the feeding grounds,

that were known to have nested ≤1 year ago in the previous season, returned only 28% positive swabs (of 11 samples) of *Acremonium* or *Penicillium* as a single isolate per positive swab.

Key to Acronyms Used in Tables 5.1.1-5.1.6.

Species
F flatback
G green
H hawksbill
L loggerhead

Nesting History
HI Heron Island
MI Milman Island
NC New Caledonia
NW North West Island
PI Peak Island
SW Swains Reef
TI Tryon Island
WI Wreck Island
U Nesting history unknown
+ Turtle had lost its original tags, so previous breeding history (if any) unknown

Table 5.1.1 Cloacal mycobiota of adult-sized and pre-gravid green sea turtles.
(maturity status established by laparoscopic examination)

Feeding Ground, Year	Tag #	Maturity Status	Cloacal Mycobiota
Heron Reef, 1996	T4289	P	Nil
	T21180	A	Nil
	T38192	P	Nil
	T78835	P	Nil
	T80233	PP	*Cladosporium* sp.
Shoalwater Bay, 1997	K8543	P	*Acremonium* sp.
	K8626	P	Nil
	K8636	P	Nil
	K8671	P	Nil
	T15157	P	Nil
	T43594	P	Nil
Heron Reef, 1997	K8864	P	Nil

Maturity Status
A mature-sized animal yet to breed, fully developed gonads but no *corpora albicantia*
P pubescent immature, partly convoluted oviduct
PP pre-pubescent immature, white, straight oviduct

Table 5.1.2 Cloacal mycobiota of feeding animals, not having bred in the past 2 years and not preparing to breed in the current season.
(breeding status established by laparoscopic examination: *corpus albicantia* present with no detection of *corpus lutea* or atretic follicles)

Location, Year	Tag #	Species	Nesting History (Rookery' Year)	Cloacal Fungi
Heron Reef, 1996	T4815	G	U	*Cladosporium* sp.
	T13971	G	NW'84,WI'84	Nil
	T95418	G	TI'96	Nil
	T32562	G	NW'87	Nil
	X22178	G	HI'84	Nil
	X9334	L	U'87'95,NC'91	Nil
Heron Reef, 1997	K3329	G	U	*Acremonium* sp.
	K8954	G	U	*Penicillium* sp.
	T6302	G	HI'84'89	Nil
	T38070	G	U'96	Nil
	X2031	L	SW'85,U'87'92'95	Nil
Shoalwater Bay, 1997	K8535	G	U	*Acremonium* sp.
	K8547	G	U	Nil
	K8551	G	U	Nil
	K8553	G	U	Nil
	K8587	G	U	Nil
	K8589	G	U	Nil
	K8605	G	U	Nil
	K8625	G	U	Nil
	K8634	G	U	Nil
	K8705	G	U	Nil
	K8713	G	U	Nil
	K8714	G	U	Nil
	T39716	G	U	Nil
	T50861	G	U'89	Nil
	T57019	G	U	Nil
	T57190	G	U	Nil
	T77432	G	NW'94	Nil
	T82783	G	HI'94	Nil
	T84070	G	WI'94	Nil

Table 5.1.3 Cloacal mycobiota of courting turtles, not having bred in the past 2 years. (captured during courtship or demonstrating obvious courtship damage)

Location, Year	Tag #	Species	Nesting History (Rookery'Year)	Cloacal Fungi
Heron Reef, 1996	T47874	G	NW'89	Nil
	T48114	G	NW'89	Nil
	T49026	G	WI'89	Nil
	T67303	G	HI'92	Nil
	T69886	G	NW'92	*Cladosporium* sp.
	T95240	G	U	Nil
	T95252	G	U	Nil
	T95255	G	U	*Cladosporium* sp.
	T95261	G	U	*Cladosporium* sp.
	T95312	G	U	Nil
	T95313	G	U	Nil
	T95321	G	U	Nil
	T95322	G	U	Nil
	T95335	G	U	*Cladosporium* sp.
	T95419	G	U	Nil.
	T95480	G	U	Nil
Heron Reef, 1997	K8861	G	U	Nil
	K8929	G	U	*Penicillium* sp.
	K8936	G	U	*Penicillium* sp.
	K8970	G	U	Nil
	K8973	G	U	*Penicillium* sp.
	K8980	G	U	Nil
	K8997	G	U	Nil
	K9000	G	U	Nil
	T30355	G	HI'86'91	*Penicillium* sp.
	T64837	G	HI'92	Nil
	T66886	G	HI'92	Nil

Table 5.1.4 Cloacal mycobiota of sea turtles captured ashore after nesting.

Location, Year	Tag #	Species	Nesting History (Rookery'Year)	Clutch # at Swab	Total # Recorded Clutches in Sampling Season	Cloacal Fungi
Heron Island, 1996	K236	G	U	6	6	*Acremonium* sp.
	K537	G	U	6	6	*Acremonium* sp.
	K550	G	U	4	5	*Acremonium* sp.
	K617	G	U	6	6	*Fusarium solani*
	K736	G	U	5	5	*Fusarium oxysporum*
	K814	G	U	5	5	*Penicillium* sp., *Acremonium* sp.
	K862	G	U	5	5	*Acremonium* sp.
	K875	G	U	5	5	*Acremonium* sp.
	K903	G	U	5	5	*Fusarium oxysporum*
	K905	G	U	5	5	*Acremonium* sp.
	K957	G	U	4	4	*Aspergillus* sp., *Acremonium sp.*
	K976	G	U	5	5	*Acremonium* sp.
	K979	G	U	5	5	*Acremonium* sp.
	K4342	G	U	5	5	*Acremonium* sp.
	K4588	G	U	3	3	*Mucor* sp.
	T47846	G	NW'89	5	5	*Acremonium* sp.
	T53979	G	HI'90	5	5	*Acremonium* sp.
	T96024	G	U	7	7	*Acremonium* sp.
	X20622	G	HI'78'84'91	6	6	*Acremonium* sp.

	X25738	G	HI'81'87	7	7	*Fusarium solani*
Wreck Island, 1998	K24303	L	U	U	U	*Fusarium solani*
	K25507	L	U	U	U	*Fusarium solani*
	K24304	L	U	U	U	Nil
	T84196	L	WI'94	≥2	U	*Fusarium solani*
	unknown	L	U	U	U	Nil
Mon Repos, 1998	K20390	L	1st Breeding Season	2	4	Nil
	K20409	L	1st Breeding Season	1	3	Nil
	K20420	L	U	2	2	*Aspergillus* spp.
	K22840	L	1st Breeding	2	2	*Aspergillus* spp.
	T14201	L	MR'84'91'94'96	5	5	*Aspergillus* sp.
	T14527	L	MR'84'86'88'90'92 '94'96	3	5	*Penicillium* spp.
	T73605	L	MR'93	2	3	Nil
	T89201	L	MR'95	2	4	*Stachybotrys* sp.
Peak Island, 1998	K17511	F	U	U	U	*Aspergillus* sp.
	T75251	F	PI'93'95'97+	U	U	Nil
	T75401	F	PI'93'94'96+	U	U	*Fusarium solani, Penicillium* sp.
	T83195	F	PI'94+	U	U	Nil
	T83205	F	PI'94	U	U	*Acremonium* sp.
	T83259	F	U	U	U	*Phialophora* sp.
	T83308	F	PI'94'97+	U	U	*Sporothrix* sp.
	T83518	F	PI'94'96	U	U	*Acremonium* sp.
	T96188	F	PI'97	U	U	*Acremonium* sp.
	T96245	F	PI'97+	U	U	*Acremonium* sp.
Milman Island, 1998	K23967	H	U	U	U	Nil

K24004	H	U	U	U	Nil
T48254	H	MI'93	U	U	Nil
T55878	H	MI'90	U	U	Nil
T72241	H	MI'92'95	U	U	Nil
T72524	H	MI'92	U	U	*Chrysosporium* sp.
T72701	H	MI'92	U	U	*Penicillium* sp.
T75067	H	MI'93	U	U	Nil

Table 5.1.5 Cloacal mycobiota of nesting turtles captured in the inter-nesting habitat between clutches.
(breeding status established by laparoscopic examination and detection of intra-oviducal eggs and large *corpus lutea*)

Location, Year	Tag #	Species	Nesting History (Rookery'Year)	Cloacal Fungi
Heron Island, 1997	K8956	G	U	*Penicillium* sp.
	T62392	G	HI'91	*Penicillium* sp.

Table 5.1.6 Cloacal mycobiota of turtles that bred in the prior season.
(breeding status established by laparoscopic examination and observation of atretic follicles)

Location, Year	Tag #	Species	Nesting History (Rookery' Year)	Cloacal Fungi
Heron Reef, 1996	X2031	L	SW'85'87'92'95	Nil
Heron Reef, 1997	K8857	G	U'97	*Penicillium* sp.
	T38068	G	U'97	Nil
	T4422	L	U'94'96	*Acremonium* sp.
	X2776	L	WR'85'87'92'96	Nil
Shoalwater Bay, 1997	K2836	G	NW'96	Nil
	K2966	G	NW'96	Nil
	K7580	G	U'96	Nil
	T32315	G	NW'87'96	Nil
	T43538	G	U'96	*Acremonium* sp.
	T57117	G	WI'96	Nil

F. oxysporum and *F. solani*, both of which are known egg mycobiota at all rookeries (see Chapter 4), were isolated from 60% of nesting turtles at Wreck Is., 20% at Heron Is., and 10% at Peak Is.. *F. oxysporum* was isolated from the cloaca and along the length of the oviduct of the dead green turtle at Raine Is..

The proportion of turtles with cloacal mycobiota was significantly different between nesting and non-nesting animals (χ^2=38.27, P<0.001, df=1). There was no significant difference in mycobiota among the four non-nesting categories (χ^2=2.65, P=0.45, df=3).

5.1.4 DISCUSSION

The fungi (*Acremonium*, *Cladosporium* and *Penicillium*) detected in feeding and courting animals have been isolated from marine habitats (see Moss 1986). Their presence in the cloaca might result from the exposure to seawater during defecation since none are true coprophiles in the classical sense (Domsch *et al.* 1980). The higher occurrence of these fungi in courting animals is probably due to transfer from the male during intromission, in a similar manner to the transmission of cloacal mites (reviewed by Pence and Wright 1998).

Of the fungi isolated from nesting and inter-nesting animals, 8 of the 9 are commonly found in soil (the genera *Acremonium*, *Aspergillus*, *Chrysosporium*, *Fusarium*, *Penicillium* and *Stachybotrys*) and/or on dead plant material (*Aspergillus*, *Fusarium*, *Penicillium*, *Phialophora*, *Sporothrix*, and *Stachybotrys*). They could be collected during any stage of the nesting procedure during which the ovipositor contacts the substrate. *Mucor* sp. are commonly found in dung lying on the ground, but not at great depth (Domsch *et al.* 1980). The guano from the large numbers of seabirds at Heron Is. (reviewed in Section 5.3) may be the source of this fungus, which could be collecting on the ovipositor during emergence or body pitting. *Aspergillus* spp. have previously been identified as mycobiota of the intestine in other reptiles, but at a very low incidence (3% of 200 animals, Gugnami and Okafor 1979), so are most likely to have also been contracted during nesting.

Once the breeding season has finished and nesting turtles return to the feeding grounds, the occurrence of cloacal mycobiota decreases. Flushing of the cloaca during urination, defecation, and passage of seawater probably removes most soil fungi accumulated during nesting. Consequently, there would seem to be a low probability of chronic fungal invasion of the oviduct leading to contamination of eggs in the next nesting season.

The isolation of *F. oxysporum* from the length of the oviduct of a dead turtle on Raine Is. provides some evidence of the potential for acute intra-oviductal infection. It is unlikely that spores were transferred the full length of the oviduct by ciliary action in the few hours elapsed since the previous night's nesting, so they must have originated from nesting activity earlier in the season. Maintenance and transport

within the oviduct could occur in the same manner as discussed below for spermatozoa.

The sea turtle oviduct may be up to 7m in length (green turtle 7m, Phillott pers.ob.; olive ridley turtle 4-6m, Owens 1980). At intromission spermatozoa are deposited at the cranial extremity of the cloaca (Saint-Girons 1975) and probably mobilised by prostaglandins of the ejaculate (Karim 1972). Their passage from the cloaca may be aided by the adovarian action of stereo-cilia on the oviductal columnar epithelium (Frye 1991) and perhaps also by oviductal motility (Harper 1982). Fertilisation occurs in the magnum (Solomon and Baird 1979) after which it is likely the remaining spermatozoa are pushed in front of the descending ova (Bobr *et al.* 1964, Hattan and Gist 1975, Owens 1980) and into storage areas until the next clutch is ovulated (Gist and Congdon 1998).

Sperm storage tubules (SSTs) of freshwater turtles have been described from the glandular region of the oviduct (Gist and Jones 1987, Gist and Jones 1989, Gist *et al.* 1990, Gist and Congdon 1998) and uterovaginal junction (Bakst 1987, Birkhead and Møller 1992, Gist and Congdon 1998). Solomon and Baird (1979) found "nests" of spermatozoa in similar locations in the green turtle. Reptilian SSTs appear as crypt-like extensions of the oviductal epithelium at the base of mucosal folds (Gist and Congdon 1998). These crypts contain eosinophilic submucosal glands similar in morphology, but differing in function, to the albumen and shell glands (Gist and Jones 1987). SSTs have no structural specialisation for spermatozoa storage (Palmer and Guillette 1988) or maintenance, and instead appear to simply be sites of residence (Gist and Congdon 1998). Spermatozoa survival within the SSTs is

possibly due to the physiologically favourable environment afforded by the oviductal mucus and protection against phagocytic attack (Harper 1982).

The means by which spermatozoa are mobilised from these crypts is unclear. One possibility is that the descending ovum, the diameter of which is greater than that of the empty oviduct, distends the oviductal wall, squeezing albumen proteins from the submucosal glands into the lumen (Palmer and Guillette 1991). Spermatozoa which fertilise the next clutch might also be mobilised from the SSTs by this distension. Presumably the remaining spermatozoa are pushed back into the SSTs by the actual passage of the ova. The surge of progesterone associated with current ovulation (Gist and Congdon 1998), intense vacuolisation in cells at the base of the SST, and/or increased osmotic pressure due to breakdown of intracellular mucopolysaccharides (Hoffman and Wimsatt 1972) are also believed to induce spermatozoa from their storage sites.

During prolonged storage spermatozoa may lose their tails, and must rely on oviductal cilia to transport them to the magnum (Frye 1991). If fungal spores or hyphal fragments lodged in SSTs cranial to the shell forming section of the oviduct, it is possible they might remain viable there for extended periods and ultimately be transported along with the acaudal spermatozoa. Such fungi could then be enclosed within the eggshell secretions, which are likely to provide an extremely favourable environment for growth.

The low occurrence of known egg mycobiota in cloacal samples (15% of all nesting turtles, and 0% of non-breeding female adults) suggests the potential for chronic inter-seasonal oviductal contamination of sea turtle eggs by these fungi is low. In contrast, the presence of *F. oxysporum* in the oviduct of a freshly dead nesting female suggests acute intra-seasonal contamination may be possible. To quantify whether such spores become incorporated internal to the eggshell would require the extensive sampling and sacrifice of eggs to analyse their contents, and so was not conducted.

5.2 THE RELEVANCE OF THE *PISONIA GRANDIS* FOREST TO FUNGAL COLONISATION OF SEA TURTLE NESTS

5.2.1 INTRODUCTION

The predominance of *Pisonia grandis* (Nyctaginaceae) on islands where loggerhead sea turtle hatch success is low led to the hypothesis that it may be a contributing factor. *P. boydii* is an opportunistic infectant described in veterinary and human medicine (Rippon 1982), but there is no record of its involvement in plant disease. However, numerous strains of *F. oxysporum* are wilt pathogens and *F. solani* may cause root rot, canker and wilts (Booth 1971). Anthracnoses in the form of leaf spots are common on many *P. grandis* at Heron Is.. If a fusaria is the causative agent, abscission would add fungi known to invade sea turtle eggs to the soil.

In its wild state *P. grandis* is almost exclusively confined to small uninhabited islands with large seabird colonies (St John 1951, Airy Shaw 1952), throughout the Indian and Pacific Oceans (St John 1951, Airy Shaw 1952, Stemmerik 1964, Hunt

1967, Cribb 1969). *P. grandis* is found on 44 Great Barrier Reef islands, most of which are in the sGBR (Walker 1991b), including all of the Capricorn/Bunker Group (Cribb 1969). A central forest is usually surrounded by natural fringing vegetation, although erosion may bring the beachfront to the forest (Cribb 1969). In addition to abundant seabirds, the presence of such forests appears heavily reliant on both a specific soil and rock base (Airy Shaw 1952, Cribb 1969) since Christophersen (1927 in Airy Shaw 1952) noted the association of *P. grandis* with richly organic, acidic, phosphatic soils overlaying a hardpan or coral conglomerate. These soils (known as the Jemo series) are typified by a dark brown to black humus formed from leaves and twigs through which calcium phosphate (from bird guano) is transported by rainwater (Fosberg 1954). Beneath is a highly phosphatic limestone (Stemmerik 1964). The calcium phosphate dissolves in the acidic conditions of the organic matter and phosphorus precipitates out when it reaches the alkaline calcium carbonate layer below, cementing the sand or gravel into a hardpan (Fosberg 1954). This edaphic condition occurs only on coral and coral debris beneath bird colonies (Stemmerik 1964) and is almost exclusive to forests dominated by *P. grandis* (Fosberg 1954).

P. grandis is often associated with islands hosting pigeons, gannets (*Sula* spp.) or noddy terns (*Anous* spp.). If the bird colonies desert an island, for whatever reason, the *P. grandis* forest disappears as it seems unable to survive without the phosphate enriched soil (Airy Shaw 1952, Stemmerik 1964) that aids germination and early development of seedlings (Airy Shaw 1952). It is believed seabirds aid in the epizoic dispersal of *P. grandis* (St John 1951) although this is disputed by Walker (1991b). At Heron Is. most fruit matures in summer when the black noddy (*A. minutus*) is nesting. Adults and fledglings are often fatally ensnared by the sticky

anthocarps fouling the body, tail or wing feathers (Cribb 1969), their carcasses presumably adding to the humus.

5.2.2 PROCEDURE

Five individual *P. grandis* showing leaf spots were examined to determine if the causative fungus was one of the *Fusarium* spp. isolated from failed eggs in sea turtle nests. Two leaves from each tree were collected and washed with sterilised, distilled water to remove bird guano and then stored in a refrigerator at 5°C. Leaf fragments (1cm^2) were surface sterilised in 1% $AgNO_3$ for 3mins and then rinsed in 5% NaCl for 1min. A final wash in sterile distilled water for 2mins removed residual silver cations. Fragments were cultured as a central inoculum on half-strength Potato Dextrose Agar at 28°C for seven days prior to examination. Dr. Roger Shivas of the Queensland Department of Primary Industries identified the isolates.

5.2.3 RESULTS

Colletotrichum gloeosporioides was isolated from all leaf fragments with anthracnoses. Plates inoculated with unblemished fragments did not show any fungal growth.

5.2.4 DISCUSSION

Colletotrichum is one of the most important genera of plant pathogenic fungi worldwide, and is especially prevalent in sub-tropical and tropical regions (Bailey and Jeger 1992). It has not been isolated from failed sea turtle eggs, so it seems unlikely *P. grandis* foliage hosts fungi likely to adversely affect sea turtle nests at Heron Is. and Wreck Is.. Previously, the only reported fungus in association with *P. grandis* at Heron Island was an unidentified basiodiomycete ectomycorrhizal

symbiont (Ashford and Allaway 1982). This fungus appears to have a limited host range or be unique to *P. grandis* (Cairney *et al.* 1994) and is unlikely to be one of the common soil saprophytes *F. oxysporum*, *F. solani* or *P. boydii* isolated from failed sea turtle eggs.

While mycoflora known to invade turtle nests are not sustained as pathogens of *P. grandis*, this does not preclude the forest from playing other roles in maintaining resident soil microbiota. Leaf litter of *P. grandis* contains significant levels of calcium oxalate. This insoluble crystalline salt of oxalic acid is synthesised by *P. grandis* as part of its pH, ionic and osmotic regulation that enables it to cope with the high pH, abundant calcium and nitrates, and lack of water of the substrate (Mills 1991). Although oxalates are toxic to many organisms, their addition to the substrate (by abscission) is unlikely to adversely affect soil microbiota since many fungi produce them as metabolic products (Arnott 1995, Franceschi and Loewus 1995). Further, substrate oxalate may actually be utilised as a carbon source by the fungi (Allison *et al.* 1995). In solution, oxalate acidifies the soil, keeping phosphorus available (see Graustein *et al.* 1977). Since phosphorus is an essential nutrient in fungi metabolism (Garraway and Evans 1984), oxalates from *P. grandis* are likely to aid fungal nutrition and maintain high soil microbiota on those islands possessing substantial stands of *P. grandis* (e.g. Heron and Wreck Islands). This is likely to contribute to the lower hatch success recorded at these turtle rookeries, and is examined in Chapter 8.

5.3 SEABIRDS AS POTENTIAL HOSTS FOR FUNGI

5.3.1 INTRODUCTION

The Capricorn Group of islands have 4 of the major colonies of black noddies (*Anous minutus*) in the Great Barrier Reef and the largest breeding population of wedge-tailed shearwaters (*Puffinus pacificus*) in the world (Hill *et al.* 1995). Colonies of both species at Heron Is. have been subject to intense surveillance.

Black noddies reside on the island year round. Breeding occurs between October and March with a peak in November/December. A single egg is laid (Kikkawa 1970) in rough nests constructed from excreta and shed *P. grandis* leaves (Barnes and Hill 1989, Hill and Rosier 1989). The majority of nesting occurs in the *P. grandis* forest, with correlations between bird density and vegetation species and physiognomy (Shipway 1969, Dale *et al.* 1984, Hulsman *et al.* 1984, Barnes and Hill 1989, Ogden 1979, 1993).

The numbers of black noddies on Heron Is. have been extensively documented (Campbell and White 1910, MacGillivray 1928, Cooper 1948, Shipway 1969, Kikkawa 1970, Bingham 1977, Ogden 1979, Hulsman 1983, 1984; Barnes and Hill 1989, Ogden 1993, Hill *et al.* 1997). From 53 nests in 1910 (Kikkawa 1970), the population increased to 63,000 ±7,000 pairs in 1992 (Ogden 1993); an almost exponential increase in the last 75 years (Barnes and Hill 1989) of *ca.* 7% per annum (Ogden 1993). This rate of increase predicted 116,615 breeding pairs by "the turn of the century" (Ogden 1993), but high adult mortality in 1997 due to lack of prey (P. O'Neill pers.comm.) has resulted in reduced numbers over the past few years

(Phillott pers.ob.). Population growth has been accompanied by an expansion of the nesting area into areas of human habitat and the coastal woodlands since the report by Shipway (1969), together with an increase in the number of nests per tree (Barnes and Hill 1989).

The migratory wedge-tailed shearwaters arrive at Heron Island in October (Campbell and White 1910, Cooper 1948, Moulton 1961, Gross *et al.* 1963, Kikkawa 1970, Bingham 1977, Ogden 1979). Nesting commences between mid-November and mid-December (Dyer and Carter 1997) and peaks in late-December (Gross *et al.* 1963). A single egg (Dyer and Hill 1990) is incubated in a burrow (Hill and Rosier 1989, Dyer and Hill 1990) or in protected hollows on the ground among tree roots (Dyer and Hill 1990). Adults and fledglings depart the island by May-June (Miles 1964, Kikkawa 1970).

Nesting by wedge-tailed shearwaters on Heron Island has been thoroughly described (MacGillivray 1928, Shipway 1969, Kikkawa 1970, Ogden 1979, Hulsman 1983, 1984, Hill and Barnes 1989, Ogden 1994, Carter *et al.* 1996, Hill *et al* 1996). Although the literature appears to report an increasing population from 8,300 burrows in 1965 (Shipway 1969) to 13,381±1,556 (Hill *et al.* 1996), Dyer *et al.* (1995) have corrected for different sampling methodology to deduce a mean of approximately 15,000 active burrows over the past 35 years.

5.3.2 PROCEDURE

The cloacal exteriors each of twenty black noddies and wedge-tailed shearwaters were swabbed to compare their mycobiota with that isolated from failed

sea turtle eggs. Adult noddies were captured while on the nest, briefly removed, swabbed and replaced. Animals with eggs or young chicks were not sampled. Shearwaters were captured by hand during the nightly courtship or pre-dawn congregations prior to leaving the island for feeding. Swabbing was performed at the point of capture and animals were released immediately.

Swabs (MW170 TRANSTUBE®, Amies Clear Transport Media) were refrigerated at 3-5°C prior to incubation. Fungi were incubated on specific media and identified according to Booth (1971), Ellis (1971), Pitt (1979), Carmichael *et al.* (1980) and Klich and Pitt (1988).

5.3.3 Results

All 20 of the *P. pacificus* and 19 of the 20 *A. minutus* swabs were positive for cloacal mycobiota. Isolates were *Penicillium citrinum*, *Fusarium semitectum*, *F. trichothecioides*, *Aspergillus unguis*, *A. candidus*, *Acremonium* sp., *Alternaria* sp. and *Drechslera* sp. (see Table 5.3.1). Most swabs produced monocultures of *P. citrinum* (78% *A. minutus*; 70% *P. pacificus*) or *F. semitectum* (5% *P. pacificus*), but mixed cultures of *P. citrinum* + *F. semitectum* (11% *A. minutus*; 10% *P. pacificus*), *P. citrinum* + *F. trichothecioides* (5% *P pacificus*), *P. citrinum* + *Drechslera* sp. (5% *P. pacificus*), *P. citrinum* + *A. candidus* + *A. unguis* (11% *A. minutus*) and *Alternaria* sp. + *Acremonium* sp. (5% *P. pacificus*) were also obtained.

Table 5.3.1 Occurrence of fungi in seabird cloacal swabs.

	Occurrence of Fungi (%)	
Fungi	*A. minutus* (n=20)	*P. pacificus* (n=20)
P. citrinum	95	90
F. semitectum	10	10
F. trichothecioides	0	5
A. unguis	5	0
A. candidus	5	0
Acremonium sp.	0	5
Alternaria sp.	0	5
Drechslera sp.	0	5

5.3.4 DISCUSSION

All of the genera isolated from seabirds are commonly associated with soil or agricultural crops (Booth 1971, Ellis 1971, Pitt 1979, Carmichael *et al.* 1980, Klich and Pitt 1988) and some (*Acremonium, Alternaria, Aspergillus*, *Fusarium*, *Penicillium*) are known to be cellulolytic fungi from birds nests (Mazen *et al.* 1994). None are true coprophiles (see Webster 1970) or keratinophiles (Pugh and Evans 1970, Rees 1977), so their most likely source is from the nesting material or the burrow substrate. None are known invaders of sea turtle eggs (see Chapter 3).

The absence of *F. oxysporum* and *F. solani* is surprising as both have been detected in wild and domestic bird nests (Mazen *et al.* 1994) and *F. oxysporum* has been isolated from feather samples (Hubalek *et al.* 1995). They are known cellulolytic fungi (Mazen *et al.* 1994) and their absence from samples taken from Heron Is. may be due to small sample size, or the lack of sampling of nesting material.

Seabirds are expected to play an accessory role in maintaining resident soil microbiota. Staunton Smith and Johnson (1995) calculated a guano deposition of *ca.* 107t from *A. minutus* and *ca.* 22t from *P. pacificus* on Heron Is. in 1992 alone. Total

annual deposition of guano is expected to contain 9.4t of nitrogen and 1.9t phosporus. While much of the nitrogen is leached by rainfall (Heatwole *et al.* 1981) the phosphorus is expected to be incorporated in to the soil and be of importance in maintaining the island vegetation (Allaway and Ashford 1984). Increased soil nutrients may also be expected to maintain high numbers of microbiota, so soil mycobiota density of Heron Is., compared to other sea turtle rookeries in eastern Australia, has been examined in Chapter 8.

CHAPTER 6

THE DISTRIBUTION OF FAILED EGGS AND APPEARANCE OF FUNGI IN SEA TURTLE NESTS

6.1 INTRODUCTION

Solomon and Tippett (1987) and Wyneken *et al.* (1988) described clusters of non-viable or diseased eggs within nests, which, as noted earlier, suggests an infectious aetiology (Wyneken *et al.* 1988). These clusters were noted during examination of natural, relocated and artificially incubated clutches of loggerhead eggs (Wyneken *et al.* 1988), and natural leatherback nests (Solomon and Tippett 1987). Fungal invasion in leatherback nests was primarily localised at the clutch centre with 68% of failed eggs (the proportion of the entire nest was unstated) occurring at the core (Solomon and Tippett 1987).

In their examination of post-emergence nests, Solomon and Tippett (1987) allocated eggs to the "top, middle or bottom" of the egg mass, but it is unlikely this description accurately reflected the actual position of the eggs during incubation. Even if no mixing occurred during nest excavation, eggshells and failed eggs are likely to have been reassorted during the scramble that occurs during hatching and emergence.

The present study utilised artificial incubation of eggs in natural arrangements, allowing direct observation of the distribution of failed eggs and the spread of fungi throughout the egg mass. Of particular interest was the time (pre- or post-mortem) at which fungi became apparent.

As discussed in Section 1.2, normally developing eggs exhibit a "white-spot" that expands across the eggshell, closely paralleling the internal development of the chorioallantois (Thompson 1985). Its chalk-white appearance initially indicates structural and physical changes of the eggshell associated with drying (Webb *et al.* 1987). This colouration is maintained throughout incubation, and is reflective of the eggshell's ability to meet the embryo's increasing respiratory gas exchange demands (Lomholt 1976, Rahn *et al.* 1979).

Eggs that fail to hatch usually appear yellow and display a loss of turgor and deterioration of the eggshell. Knowledge of the time lapse between embryonic death and the occurrence of physical alterations in the visual appearance of eggs is unknown, but necessary to attribute embryonic death to events such as fungal colonisation. In order to catalogue the post-mortem changes to egg appearance, embryo death was deliberately induced under controlled conditions.

6.2 PROCEDURE

6.2.1 Time Elapsing Between Embryo Mortality and Changes in the Physical Appearance of Sea Turtle Eggs

Two successive mid-clutch eggs were each collected from the ovipositor of five individual green turtles nesting at Heron Is.. Eggs were incubated at ambient laboratory temperature at the Heron Island Research Station as matched pairs, with each pair in a separate transparent plastic container (16cmx10cmx7cm) covered in oxygen permeable plastic film. Eggs were set on a 2cm substrate of autoclaved sand collected at a depth of 55cm (average nest depth) from the nesting beach. A sub-

surface trickle irrigation of sterile water maintained sand moisture to a visual standard, pre-determined by the "pinch method" of Blanck and Sawyer (1981).

Eggs were observed until the white spot covered approximately half of the surface, indicating embryonic viability (7-10 days). Eggs were then removed, weighed and replaced; one in its original orientation and the other inverted. Inversion of green (Parmenter 1980) and loggerhead (Limpus *et al.* 1979) sea turtle eggs at this time during incubation is known to cause embryo mortality. Control (uninverted) eggs were incubated until they had hatched, whereupon experimental (inverted) eggs were opened to determine the embryonic stage at which mortality had occurred.

6.2.2 The Distribution of Failed Eggs and Appearance of Fungi

The patterns of egg failure and colonisation by fungi cannot be observed in a natural nest during incubation, and interpretation of egg position after nest excavation can be criticised. It was decided the artificial incubation of eggs, in an arrangement that closely represented that of the natural nest, was the best method to directly observed fungal colonisation during incubation.

Five clutches each of green (from Heron Is.) and loggerhead (from Mon Repos Conservation Park) turtle eggs were incubated at the Heron Island Research Station in the 1997/98 nesting summer. Eggs were collected (using gloved hands) directly from the ovipositor into sterile plastic bags to reduce the likelihood of contamination. During transport of loggerhead turtle eggs, embryonic diapause was maintained by temperature depression to 8°C (Harry and Limpus 1989). Upon arrival at the

laboratory, eggs were handled according to Limpus *et al.* (1979) to prevent movement-induced mortality.

Eggs were incubated in clear, sterile, cylindrical plastic containers (22cm diameter × 15cm height) upon a 3cm deep substrate of untreated Heron Is. sand collected from 55cm (the average nest depth). Sand was obtained from beneath vegetation in areas where fungal invasion of nests was known to be high. Substrate hydric conditions were maintained by a sub-surface trickle irrigator, with sterile water, to conditions consistent with that of the "pinch method". Containers were covered with plastic film to enhance humidity yet still allow gas transfer. Depending on egg size, 50-70 eggs from a single clutch were placed in each container, mimicking the shape and arrangement of the egg mass within natural nests, except that only the bottom layer of eggs was in direct contact with the sand substrate.

Eggs were incubated at ambient temperature (Mean±SD: min. 24±1°C, max. 31±1°C) and examined daily to monitor viability, incidental mortality and appearance of fungi on their exterior. The arrangement of eggs allowed all but the middle 2-3 eggs to be observed (eggs on the distal side of the container were examined using a mirror). Each egg had a unique position within the "nest" according to sector (1-12 clockwise from a consistent starting point), stratum (1 contacting the lower substrate to 4 or 5 at top of clutch) and radius (1 at the periphery to 3 or 4 in the centre).

The exteriors of 2-3 failed eggs per clutch were swabbed with MW170 TRANSTUBE®, Amies Clear Transport Media 10 weeks after the commencement of

incubation, and samples refrigerated at 3-5°C prior to analyses. Fungi were incubated on half-strength Potato Dextrose Agar with 0.05g L^{-1} chloramphenicol and cultures compared with those described in Chapter 4. At the same time failed eggs were opened to stage the embryos according to Miller (1985).

6.3 RESULTS

6.3.1 Time Elapsing Between Embryo Mortality and Changes in the Physical Appearance of Sea Turtle Eggs

During the 7-10 days prior to manipulation, pairs of eggs showed similar signs of development in terms of progression of white spot and weight gain. White spot development ceased immediately after inversion of 4 of the 5 eggs. In the exception, the white spot continued to expand, but at a slower rate than its uninverted partner for a further 84hrs, after which expansion ceased. Data for this egg were subsequently discarded since although it did not hatch, the exact time of embryonic death was uncertain. Sixteen hours after rotation, the 4 remaining inverted eggs displayed a pale yellowing tinge to the area not encompassed by the white spot. This colour increased in intensity as time progressed and spread downwards until the entire egg displayed a distinct yellowing after 20-24hrs. The chalkiness of the white spot faded noticeably after 44hrs and shell pliability increased slightly. None of the non-rotated eggs displayed any alteration in eggshell appearance other than normal expansion of the white spot, and there was no change in shell pliability.

All of the non-rotated eggs produced hatchlings, while none of the inverted eggs hatched. When opened, the 4 inverted eggs revealed development consistent

with embryonic Stage 18-19 (after Miller 1985), suggesting death synchronous with egg rotation.

6.3.2 The Distribution of Failed Eggs and Appearance of Fungi

Both *F. solani* and *P. boydii* were isolated from the exterior of failed green and loggerhead turtle eggs (see Table 6.1). The exterior of the eggshell turned black, indicative of fungal presence. The albumen of failed eggs was typically opaque or discoloured a dull green or grey, probably caused by post-mortem bacterial growth. Albumen from viable eggs is invariably translucent.

Table 6.1 Fungi isolated from artificial green and loggerhead turtle nests.

Species	Clutch #	Nest Mycobiota
Green	1	*P. boydii*
	2	*F. solani*
	3	*F. solani* + *P. boydii*
	4	*P. boydii*
	5	*F. solani*
Loggerhead	6	*F. solani* + *P. boydii*
	7	*P. boydii*
	8	*F. solani*
	9	*F. solani*
	10	*F. solani*

The first appearance of fungi within a clutch was always on an egg that showed the signs of non-viability described in Section 6.3.1. Fungi did not appear sooner than a week after observed egg mortality. Hyphae from the failed egg then spread to the exterior of adjacent eggs. Daily observations over an 8 week period showed expansion at a rate of 1-2 eggs/day, and the entire egg mass was colonised within 10-14 days of the first appearance of fungi. Eggs that appeared healthy (i.e. white and

turgid) did not show external signs of fungal growth until it had spread from an adjacent egg.

In some clutches, a number of eggs died before fungi became apparent on one of them (see Table 6.2). The location of the initial failed egg invaded by fungi was never at the centre of the artificial clutch (see Table 6.2). Chi-square analyses showed no significant difference in the location (radius χ^2=0.00106, df=1, P=0.974; level χ^2=5.757, df=3, P=0.124) of failed eggs that became the focus for fungal invasion and those that did not, but these findings should be viewed cautiously due to the small sample sizes. Sample sizes for sector were too small to allow statistical analysis.

Table 6.2 Distribution of failed eggs within artificial sea turtle nests (numbers are percentages within each category).

Location		Initial Fungal Focus	Other Failed Eggs
Radius	1	70	62.5
	2	30	37.5
	3	0	0
	4	0	0
Level	1	0	25
	2	20	0
	3	30	25
	4	50	50
Sector	1	0	6
	2	10	6
	3	0	13
	4	10	6
	5	10	6
	6	0	13
	7	10	6
	8	20	0
	9	20	6
	10	0	6
	11	0	19
	12	20	13

6.4 DISCUSSION

6.4.1 Time Elapsed Between Embryo Mortality and Changes in the Physical Appearance of the Sea Turtle Egg

In the green sea turtle, egg inversion and subsequent embryonic death resulted in alterations to egg appearance. As eggshell structure is similar in green (Solomon and Baird 1976, Baird and Solomon 1979), leatherback (Solomon and Watt 1985, Chan and Solomon 1989), olive ridley (Acuña-Mesén 1984, Sahoo *et al.* 1996a, 1996b), Kemp's ridley (Packard *et al.* 1982, Hirsch 1983), hawksbill (Acuña-Mesén 1989) and flatback (see Chapter 11) sea turtle eggs, post-mortem changes in appearance may also be similar. Although Carthy (1992) described pore-like structures on the inner surface of loggerhead turtle eggshell membrane, these were not detected by Packard *et al.* (1982) or Schleich and Kästle (1988). Therefore, loggerhead eggshell is assumed to display similar alterations to visual appearance to that described here.

Blanck and Sawyer (1981) describe two temporary extra-embryonic membranes present during the first $2^1/_2$ weeks of development (approximately the 1st trimester of the 57 day incubation period) in loggerhead turtle eggs incubated at 28°C. The posterior amniotic tube and the "attachment membrane" (an extension of the amnion that fuses with the chorionic membrane adjacent to the eye region) aid in positioning and maintaining the embryo at the top of the egg. The physical support role of the extra-embryonic membranes is superseded after the 1st trimester by the thickening of the yolk stalk and increase of the chorion/shell membrane adherence area.

Prior to this time, egg inversion results in the disruption of egg contents and tearing of extra-embryonic membranes and blood vessels (Blanck and Sawyer 1981). Miller (1982) attributes movement induced early embryo mortality to rupturing of the vitelline membrane. The embryo remains attached to the shell, but is sheared from the embryonic disc (Ferguson 1985), allowing yolk and fluids within the vitelline sac and sub-germinal space to mix with the albumen (Ewert 1979). Post-mortem discolouration of eggs would result from this mixing. In this study, yellowing was first observed at the current northern pole, spreading toward the equator and finally encompassed the entire egg, which was probably due to re-arrangement of the egg contents after egg inversion. As the yolk re-migrates through the albumen and rearranges itself to its preferred orientation, maximal leakage of the contents of the vitelline sac would occur at the new top surface of the egg, hence the initial site of yellowing. Since the chalkiness of the white spot is caused by shell dehydration (and subsequent opacity) maintained by embryonic metabolic activity, it would fade soon after embryonic death.

The loss of turgor by non-viable eggs occurs as they lose their water holding capacity (Ewert 1979), potentially acting as water donors to adjacent viable eggs, as suggested for yolkless leatherback eggs (Hall 1990). Viable eggs maintain a water potential of -900 kPa (Ackerman 1991). Death would result in the loss of metabolic sustenance and a water potential closer to the substrate, allowing moisture transfer from the egg to the nest environment. Alternatively, water bridges may form between non-viable and adjacent viable eggs and allow direct exchange.

Unlike non-viable eggs in natural nests examined at full term, the experimentally killed eggs did not exhibit severe flaking (exfoliation) of the shell. The slight increase in pliability that occurred was probably due to loss of turgor, but may also have been indicative of post-mortem degradation of the shell structural integrity. Sea turtle eggshell consists of variable sized aragonite crystals that are not organised into individual shell units with intervening discrete pores. Instead, there are numerous open spaces allowing gas and water exchange (Solomon and Baird 1976, Baird and Solomon 1979, Packard *et al.* 1982, Schleich and Kästle 1988, Solomon and Watt 1985, Chan and Solomon 1989, Sahoo *et al.* 1996a, 1996b). When the underlying shell membrane decays post-mortem, it dissociates from the calcareous crystalline eggshell (Hirsch 1983), probably leading to the subsequent degradation of the eggshell. It is possible that the relative lack of soil microbiota (when compared to that of the natural nest) was not sufficient for complete flaking of the eggshell to be observed in this experiment. In contrast to failed eggs, flaking of viable eggs in the week prior to hatching (Miller 1982) is a result of calcium mobilisation from the eggshell by the rapidly maturing embryo (Simkiss 1962).

Eggs inverted during development showed two major alterations: yolk displacement and embryonic death. It is unclear whether changes in eggshell appearance were due to these occurrences individually, or in combination. However, other laboratory experiments (reported later in Chapters 9-10) have shown that eggs dying without human intervention present similar signs of faded chalkiness/yellowing and increased shell pliability. Yellowing is probably due to mixing of the yolk and/or sub-germinal fluids with the albumen in combination with embryo autolysis. It would normally occur in a non-inverted egg following

breakdown of the vitelline sac by protein degradation or microbial action, and be observed as a gradual stain of the entire egg rather than initiating at either pole. Fading chalkiness due to rehydration of the shell interstices as metabolic activity ceases is likely to be a direct result of embryonic death rather than inversion, and hence is a better indicator of egg mortality.

The delay between embryonic death and the physical alterations in eggshell appearance is important when interpreting results derived from artificial incubation of sea turtle eggs. Unless the white spot has faded substantially within 48hrs of a particular incubation event or mishap, egg death should not be ascribed to that event with any certainty.

6.4.2 The Distribution of Failed Eggs and Appearance of Fungi

The pattern of egg mortality and spread of fungi through the egg mass suggests an initial egg failure, allowing opportunistic invasion by soil mycobiota. In the absence of stressful environmental conditions, initial failure of the "host" egg is probably due to natural developmental abnormalities. Egg chalkiness begins to fade within 48hrs of known embryo mortality. Therefore, it does not seem likely that fungal invasion killed the initial egg, otherwise physical changes in the egg and the appearance of fungi would have been near synchronous.

The initial appearance of fungi on eggs toward the periphery and top of the clutch is consistent with this area having the highest egg mortality in this experiment. Since experimental preparation of the nest substrate is likely to result in the distribution of spores throughout the container, contamination of these eggs may

have occurred as spores fell from the underside of the plastic film and/or sides of the incubation container.

Having colonised the initial (naturally) failed egg, the fungi produced hyphae that spread to adjacent eggs, quickly killing them (as embryonic stages at death were consistent with the timing of fungal sulliage). Although conducted in an artificial environment, the results from this study are likely to correspond to events occurring within *in situ* nests.

In this experiment none of the eggs covered with hyphal growth hatched. The possible causes of embryo mortality in fungal affected eggs are investigated in Chapter 7.

At Heron Is., reduced hatching of loggerhead and green nests is associated with fungal invasion (Limpus *et al.* 1983d, see also Chapter 3) but the mortality is rarely as dramatic as in this laboratory study. The percentage of the clutch destroyed in natural nests may be influenced by the timing of the first failed egg within the incubation period and subsequent time available for fungal colonisation, the rate of hyphal spread, and the number of eggs that naturally fail and become starting points for fungi.

CHAPTER 7

POTENTIAL CAUSES OF EMBRYO MORTALITY FOLLOWING FUNGAL INVASION OF THE SEA TURTLE EGG

Solomon and Baird (1980) describe three possible mechanisms for debilitation of the developing embryo once fungi have invaded a viable egg: impeded gas exchange as the hyphae interweave with the porous eggshell matrix; transfer of fungal spores from the allantois to the embryonic tissue; and, calcium depletion of the eggshell resulting in retarded embryonic development. Kunert *et al.* (1993) reported that invasion of snake eggs (*Elaphe guttata*) by *F. solani* caused malnutrition and retarded growth or death of the embryo. Exposure to mycotoxins is another potential cause of embryo mortality that has not been considered.

7.1 THE INFLUENCE OF A DIMINISHED RESPIRATORY SURFACE AREA ON SURVIVAL OF SEA TURTLE EMBRYOS

7.1.1 INTRODUCTION

Whilst physical and physiological defences (such as eggshell structure and anti-pathogenicity of albumen) play an important role in protecting the turtle egg from the biotic nest environment, inter-specific variation in egg size may result in differences among turtle species in response to a similar sized area of fungal growth and subsequent impediment of respiratory surface area.

After oviposition, egg yolk migrates to the upper (north) pole because of differences in specific gravity between it and the albumen (Fisk and Tribe 1949).

This results in the embryonal region of the blastoderm, which is associated with the yolk, closely contacting the shell membrane (Fisk and Tribe 1949) and presumably aiding respiration (Blanck and Sawyer 1981). Dehydration of the eggshell and albumen adjacent to the embryo results in formation of the sub-embryonic fluid and an opaque white spot on the shell that progressively encompasses the originally translucent egg (as reviewed in Section 1.2).

Opacity of the white spot occurs due to changes in the optical (Webb *et al.* 1987) and (possibly) structural (Ferguson 1982) properties of the eggshell, although the latter has yet to be conclusively demonstrated in sea turtle eggs. Dehydration of the shell and albumen not only allows formation of the sub-embryonic (Ewert 1979, 1985, Webb *et al.* 1987) and allantoic (Manolis *et al.* 1987) fluids, but is necessary for a gaseous connection between the nest atmosphere and the respiratory exchange surfaces within the egg itself (Lomholt 1976, Rahn *et al.* 1979). An increase in oxygen conductance precedes embryonic oxygen requirements, as the entire egg appears white by day ten at optimal temperatures (pers.ob.), while maximum oxygen demand does not occur until the second half of incubation (Ackerman 1981b).

Convective gas exchange in avian and reptilian eggs is prevented by small pore size and the shell architecture (Rahn *et al.* 1971, Wangensteen 1972, Rahn *et al.* 1979, Paganelli 1980). At oviposition, simple diffusion may be obstructed by the eggshell, shell membranes, chorio-allantois (Rahn *et al.* 1979) and the fluid-filled eggshell matrix (Deeming and Thompson 1991). As the diffusion co-efficient of oxygen through air is approximately 10^4 greater than that through water (Black *et al.*

1984), some of the eggshell liquid must be removed from the pores and shell membrane to increase oxygen permeability (Lomholt 1976).

In contrast, the flow of liquid water is faster through liquid- than air-filled pores (Thompson 1985). Consequently the water and oxygen uptake requirements of the developing embryo have competing needs for water-filled and air-filled eggshell interstices. Some flexible-shelled eggs require environmental water to allow the embryo to totally mobilise yolk resources (Packard and Packard 1980, Packard *et al.* 1977, 1981) and maximise hatchling size (Packard *et al.* 1981, 1983). Therefore most liquid water uptake should occur in the first days of incubation (Miller 1996) before complete development of the white spot, by which time most shell fluid has been removed. Whilst Deeming and Thompson (1991) divide the egg into respiratory (opaque shell) and water exchange (translucent shell) domains, gaseous water exchange should still be possible across the entire shell surface.

7.1.2 PROCEDURE

Flatback and green turtles were selected to investigate the influence of egg size on embryo mortality after respiratory surface disruption, as there is considerable variation in egg size between these species (see Section 1.2). Loggerhead turtle eggs were not used due to their higher listing on the state fauna register (Nature Conservation (Wildlife) Regulation 1994).

Eight eggs were collected directly from the ovipositor from each of five flatback turtles from Peak Is. and green turtles from Heron Is.. All eggs were within the normal range of diameter and weight for each species. Flatback turtle eggs were

incubated in a single styrofoam box on a 5cm deep substrate of moist sand from the nesting beach at Peak Is.. The box was buried to beach level with a further 5cm layer of sand placed on top of the lid to act as a thermal buffer. Humidity within the box was maintained by open flasks of fresh water. Green turtle eggs were incubated individually in plastic containers (12cm diameter, 7cm height) on a 3cm deep substrate of moist sand from the nesting beach at Heron Is. at ambient laboratory temperature at the Heron Island Research Station. The tops of the containers were covered with oxygen permeable plastic film to maintain humidity.

After 9 days of incubation (approximately 10-15% of the incubation period at extant temperatures) the eggs from each clutch were randomly but equally allocated to eight treatments: unhandled (control); 25%, 50% or 75% (commencing at both north and south poles) and 100% of the eggshell painted with a thin coating of white petroleum jelly (Vaseline®). Treatments were established to exclude gas exchange over different proportions of the egg surface, on both respiratory exchange (i.e. chorio-allantoic) and non-respiratory exchange domains. After a further 2 days of incubation, the eggs were opened and embryos inspected for viability, which was determined by the presence of heart contractions.

7.1.3 RESULTS

In both species 9 days of pre-treatment incubation at ambient temperature resulted in the white spot encompassing approximately half of the egg. All treatments covering the northern pole with Vaseline® caused immediate cessation of white spot enlargement. In the remaining treated eggs there was no progression of the white spot into southern areas where Vaseline® had been applied.

There was an obvious decrease in embryo survival as the respiratory surface area was reduced (see Figures 7.1.1 and 7.1.2). These differences depended on species, as well as the proportion and location of treatment. In both species, 100% of control eggs survived, indicating that handling procedures during collection and experimentation had no influence upon embryo viability. Coverage of the north pole quartile of the egg reduced survivorship to 40% in flatback and 0% in green turtles. Mortality increased in flatback turtles with area of inhibition covered with Vaseline®, until 0% survivorship at 75% coverage from the north.

Figure 7.1.1 Flatback Turtle Embryo Survival After Respiratory Surface Disruption.

(shaded area represents the surface inhibited by Vaseline®)

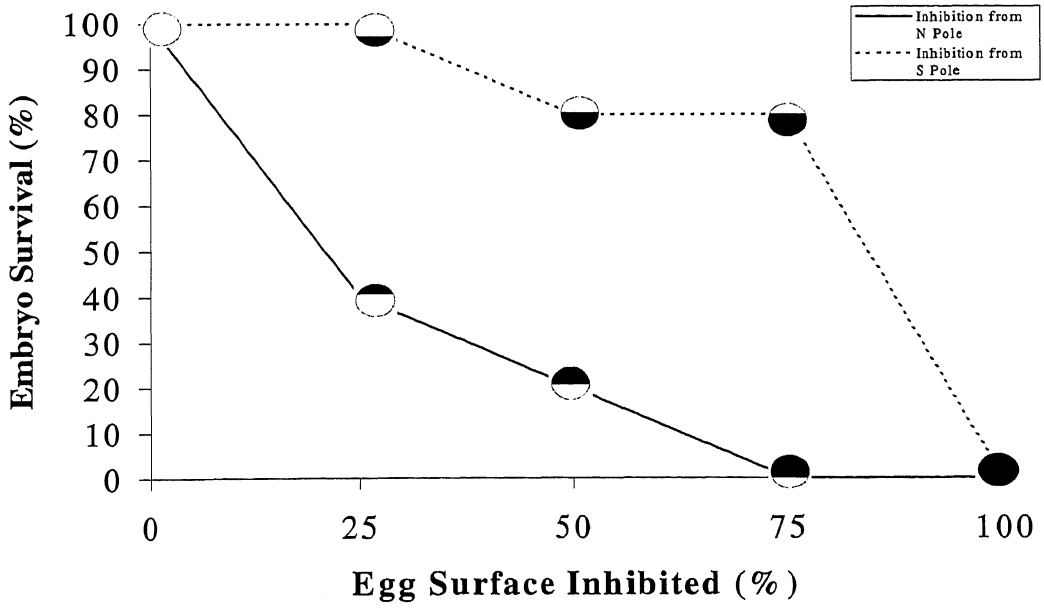
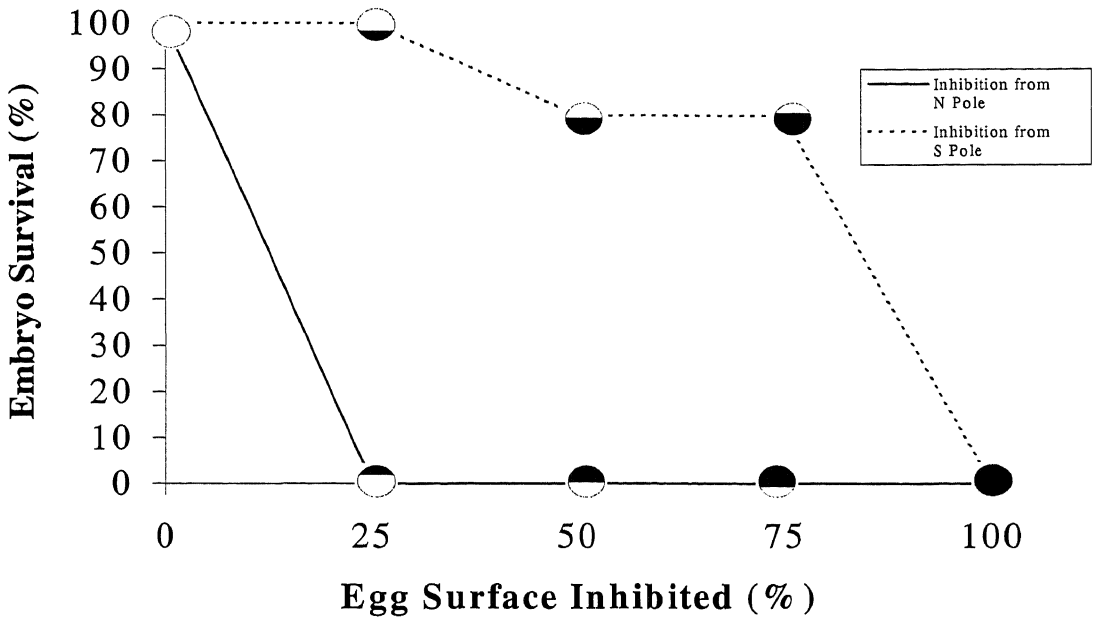


Figure 7.1.2 Green Turtle Embryo Survival After Respiratory Surface Disruption.
(shaded area represents the surface inhibited by Vaseline®)



Progressive coverage from the south pole affected the embryos of both species in the same manner. There was no mortality until the white spot area was impinged upon. Total coverage of the eggshell with the petroleum jelly resulted in 0% embryo survival.

7.1.4 DISCUSSION

Although experiments were confounded in location, time and minor differences in method, the obvious difference in mortality following inhibition of the northern/southern hemispheres can be attributed to embryo position and the extent of the white spot. Any impedance to gas exchange in the northern (embryonal) area reduces egg survival, although there appears to be inter-specific variation in susceptibility. Increasing coverage of southern areas of the eggshell with Vaseline® did not decrease survivorship until the northern quartile was compromised. The clear

inference is that mortality was due to hypoxia/anoxia through reduction of the embryo's gas exchange surface. Alienation of gas exchange function across the southern hemisphere of the egg may cause mortality later in incubation, when increased oxygen demand requires the functional white spot to encompass all or most of the egg surface.

The white spot never expanded into areas covered with Vaseline®. This may have indicated immediate embryonic death (and cessation of chorio-allantois membrane attachment to the eggshell), or simply that the petroleum jelly reduced the membrane/shell adherence mechanism and/or prevented absorption of fluid from the eggshell matrix which normally results in the egg whitening (Thompson 1985, Webb *et al.* 1987).

For the green sea turtle, any shell interference proximal to the embryo resulted in death. Unpublished results suggest that inhibition of an area as small as $1cm^2$ above the gastrula will arrest further development in green turtle eggs (C.J. Limpus, pers. comm.). However, a number of flatback turtle embryos survived coverage of the upper quarter and half of the egg, suggesting the uncovered remainder of the white spot (which is larger than that of green turtle eggs) was sufficient to sustain the embryo in the early stages of development. While little is known about embryonic mass specific (or stage specific) metabolic requirements, the larger flatback embryos may have a lower relative oxygen demand, allowing survival under more extreme conditions.

Exposure to unnaturally depressed oxygen levels in underground nests may be caused by microbial respiration (Seymour *et al.* 1986). Regardless of the cause, hypoxia in sea turtle nests is likely to retard embryonic growth and development, in turn affecting hatch success, hatchling mass and incubation period (Ackerman 1981a, Kam 1993, Kam and Lillywhite 1994). Hatchling fitness may be adversely influenced if hypoxia is experienced early in development; as an example, exposure of chicken eggs to 6 hrs of hypoxia caused cardiovascular malformations (Jaffee 1974) whilst 5 days of hypoxia resulted in lowered red blood cell counts and depressed embryonic mass (Ackerman and Ramm 1971). However, as oxygen consumption increases sigmoidally over time during sea turtle egg incubation (Ackerman 1981b), in tandem with embryonic growth (Ackerman 1981a), developmental stages involving rapid growth are likely to be more affected by hypoxia than those during differentiation (Kam 1993).

Whilst it has not been conclusively demonstrated that microbial invasion of sea turtle eggs impedes gas exchange, it may be a factor contributing to the known mortality in the presence of mycoflora. Should fungal hyphae significantly impede oxygen passage across sea turtle egg shells, it is unlikely they would be as effective as the Vaseline® applied in this experiment, so embryo mortality would not be as dramatic. Even so, the differing survival of species with large and small eggs when oxygen deprived, as demonstrated in this experiment, may contribute to inter-specific variation in natural embryo mortality.

If fungal invasion of sea turtle eggs impedes gas exchange, the site and proportion of coverage will have a major effect on embryo mortality. During the early stages of incubation, inhibition of the eggshell adjacent to the embryo reduces

survivorship dramatically. This is compounded by egg size, which determines the gross amount of unaffected respiratory area. Given that fungal growth on an eggshell can reduce oxygen exchange capability, the mode of invasion becomes crucial to embryo survival. Mortality of eggs in a clutch during early stages of incubation would be much higher if the fungal introduction were from spores falling down from the covering beach sand, rather than by growth upwards or laterally from surrounding sand. This effect would predominantly establish fungal colonies on the more critical upper (northern) domain for gas exchange.

Differences in species susceptibility may be further confounded by weight and/or stage specific metabolic demands of the embryo. Denial of gas exchange capability across the southern hemisphere of the egg has a lesser effect, reducing survivorship only when the white spot is impinged upon. While denial of respiratory exchange surface is presumed to have been the cause of mortality in this experiment, it is possible that some other inhibition of white spot function and/or development may have been operating.

7.2. THE POTENTIAL FOR CALCIUM DEPLETION OF EGGSHELL AFTER FUNGAL INVASION OF SEA TURTLE EGGS

7.2.1 INTRODUCTION

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, so that it must also be mobilised from the eggshell in 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.* 1996a).

Sea turtle eggshell consists of two layers: an outer inorganic surface (composed of organised, crystalline aggregates of calcium carbonate in the aragonite form– Solomon and Baird 1976) and an underlying organic component (the shell membrane or *membrana testacea*, consisting of dense fibrous organic matter– Packard and Packard 1979, Sahoo *et al.* 1996a; with a thin basement membrane– Sahoo *et al.* 1996a). There is no cuticle on the egg exterior, and no distinct pore structure (Solomon and Baird 1976). The eggshell is the egg's first defence against microbial invasion, so it is of great importance.

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

7.2.2 PROCEDURE

To estimate the initial calcium concentration of sea turtle eggshells, one egg was collected directly from the ovipositor from each of five flatback (from Peak Island), loggerhead (from Mon Repos), hawksbill (from Milman Island) and green (from Heron Island) sea turtles. 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 then 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 $10\times10\mu$m at $\times1000$ magnification, for 60 seconds. Five fragments from each shell were analysed for the percentage concentration of calcium. Statistical analysis of eggshell calcium content among species was carried out using a one-way nested analyses of variance (with eggs as a nested factor within species).

To determine the calcium content of eggshells affected by fungus, five eggs from a single clutch of a green sea turtle (that had been collected for other research purposes) were analysed for shell calcium. The clutch was collected at Heron Island, and stored at 8°C for 48 hours at the Heron Island Research Station 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 on a natural sand substrate, indicating

nil 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 analysed as described previously. Statistical analysis of eggshell calcium content among eggs with and without fungi was carried out using a one-way nested analyses of variance (with eggs as a nested factor within fungal presence/absence). 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).

7.2.3 RESULTS

The calcium contents of eggs from each species are given in Table 7.2.1. There was no difference in calcium concentration of the eggshell organic or inorganic component among species (organic: $F_{3,16}$= 1.52, P>0.05; inorganic $F_{3,16}$=1.12, P>0.05) or among the eggs nested within each species (organic component $F_{16,80}$= 1.517, P>0.05; inorganic component $F_{16,80}$= 1.045, P>0.05). Growth of *F. solani* on the exterior of green sea turtle eggs (see Table 7.2.2) significantly reduced the calcium content of the outer, inorganic layer, but did not alter that of the inner, organic membrane (nested ANOVA: inorganic component $F_{1,8}$=11.51, P=0.023; organic component $F_{1,8}$= 0.02, P>0.05). There was no difference amongst eggs nested within the 2 categories (inorganic component $F_{8,40}$= 1.197, P>0.05; organic component $F_{8,40}$= 1.263, P>0.05). S.E.M. imagery of the fragments during calcium analysis indicated colonisation 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 egg are given in Table 7.2.3.

Table 7.2.1 Calcium content of hawksbill, loggerhead, flatback and green turtle eggshell (n=5 for each species).

	Calcium Content of Eggshell (%)			
	Organic Component		Inorganic Component	
Turtle	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

Table 7.2.2 Calcium content of non-affected and fungal-affected green turtle eggshell (n=5 for each species).

	Calcium Content of Eggshell (%)			
	Organic Component		Inorganic Component	
Fungal Presence	Mean	SD	Mean	SD
Yes	4.84	4.46	33.36	5.97
No	4.66	4.23	40.09	7.03

Table 7.2.3 Calcium concentration of *F. solani* removed from the exterior of green turtle eggs.

	Calcium Content %		
Egg #	Mean	SD	n
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
Mean	3.20	2.01	n=5 eggs

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) compared with 40.08% actual.

7.2.4 DISCUSSION

This is the first report of calcium analyses of hawksbill, loggerhead and flatback sea turtle eggshell. Solomon and Baird (1976) reported calcium to be 20% of green turtle eggshell, but their analysis was performed on homogenised eggshell that included both the organic and inorganic components. Calculating the results of the present study in the same fashion gave an average of 19.15% calcium for green turtle 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, (see high SD values in Table 7.2.1), there was no visual evidence of amorphous calcium deposits attributed to "calcium splash". This eggshell phenomenon occurs in birds after delayed oviposition (often due to stress), resulting in irregularly distributed superficial and superfluous calcium (Chan and Solomon 1989). In sea turtles, delayed oviposition may occur after disturbance during nesting (by humans or other turtles), or through failure of nest construction due to an unsuitable substrate. However, this is unlikely to result in calcium splash because shell formation is already complete and the eggs are downstream of 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 any egg.

F. solani is one of three species of fungi regularly identified from failed eggs of turtles nesting in eastern Australia (see Chapter 4) and has been implicated in embryo mortality (see Chapter 6). Its source was probably the natural sand substrate on

which the eggs were incubated (see Chapter 5.4). Since there was no post-oviposition embryonic development, the calcium loss demonstrated by eggs affected by fungus has been 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 colonised by fungi.

There has been one report of calcium loss from the eggshell of undeveloped eggs. Bilinski *et al.* (2001) recorded a loss of 25.7% of eggshell calcium from leatherback sea turtles, predominantly in the first 30 days of incubation, and a 70% increase in yolk and albumen calcium, the majority of which occurred after day 30. However, the report is fraught with apparent calculation errors and lacks details of procedure, sample size etc.. For example, Bilinski *et al.* (2001) stated that freshly oviposited eggs contain 1.23±0.43g calcium per egg. This calcium was contained in the eggshell (412.92mg calcium gdw^{-1} x 2.10g dry weight = 867.132mg calcium) and yolk and albumen fraction (7.01mg calcium gdw^{-1} x 12.70g dry weight = 89.027mg calcium). The addition of the calculated eggshell and yolk/albumen calcium contents (956.159mg or 0.956g calcium) does not equal that of the reported egg calcium (1.23±0.43g calcium per egg). This is probably the result of a procedural error where different eggs were used for the total egg and component (eggshell, yolk/albumen) calcium analyses. Another miscalculation occurs in the determination of hatchling calcium and its source. Bilinski *et al.* (2001) stated hatchlings gained 941.8mg calcium by the time of hatching. This calcium was derived from the eggshell (42.9% or 0.429 x 412.92mg calcium gdw^{-1} x 2.10mg dry weight = 372.000mg calcium) and yolk and albumen fractions (20.8 % or 0.208 x 7.01mg calcium gdw^{-1} x 12.70g dry weight = 18.518mg calcium). Again, the stated

hatchling calcium (941.8mg) is not equal to the accumulative values for eggshell and yolk/albumen calcium (390.5mg calcium).

Bilinski *et al.* (2001) does not state the number of eggs analysed at each developmental stage to determine eggshell calcium loss in undeveloped eggs, and these cannot be determined from the text or figures of the paper. In addition, the natural beach sand on which eggs were incubated was not heat-sterilised and would contain substrate mycobiota. Bilinski *et al.* (2001) did not described fungus on the exterior of the failed eggs used for calcium analysis, but it is possible external mycobiota may have contributed to the loss of calcium from the sea turtle eggshell used in their study. Considering the apparent problems with the accuracy and experimental validity of the procedures reported by Bilinski *et al.* (2001) their findings regarding eggshell calcium loss in undeveloped eggs have to be viewed with extreme caution. Consequently, the calcium loss from green turtle eggshell in the present study has been ascribed to fungus on the egg exterior.

The similarity of eggshell calcium levels in this study and the eggshell structure (see Chapter 11) of the 4 turtle species investigated in this study suggests fungal utilisation of calcium is likely to be similar across all species of sea turtle. As the fungus was superficial on the egg and did not reach the shell membrane, it is not surprising that there was no calcium diminution of the latter. Analysis after a longer period of fungal utilisation might show decreased levels of shell membrane calcium if the eggshell were eventually penetrated by fungi.

Two other species of fungi (*F. oxysporum* and *P. boydii*) have been commonly isolated from failed sea turtle eggs in eastern Australia (see Chapter 4). 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) with it being regarded as both a macro- (Garraway and Evans 1984) and micro- (Jennings and Lysek 1996) nutrient. Calcium 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. As 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 may 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 mobilisation 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.* 1996a) as a result of calcium depletion (Sahoo *et al.* 1996a). Premature loss of eggshell integrity, due to fungal secondment of calcium, would weaken its function as a physical 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 is likely to result in embryo mortality.

7.3 PENETRATION OF THE EGGSHELL AND INVASION OF EMBRYONIC TISSUE BY FUNGI

7.3.1 INTRODUCTION

To utilise embryonic tissue as a source of nutrients, fungi must first penetrate the inorganic and organic layers of the eggshell since the lack of pores prevents direct entry of hyphae and spores from the exterior. Kunert *et al.* (1993) suggested strong proteolytic and lipolytic activity would allow fungi to penetrate the eggshell. A typical strain of *F. solani* invading snake eggs (Kunert *et al.* 1993) and the plant pathogen *F. oxysporum lycopersici* (Hankin and Anagnostakis 1975) have demonstrated some of these capabilities (not all were investigated). It is not known if *P. boydii* produces similar hydrolytic enzymes, or if the *F. oxysporum* and *F. solani* isolated from sea turtle eggs produce the same enzymes as other strains. Positive activity of the egg mycoflora on appropriate media would indicate the likelihood of *F. oxysporum*, *F. solani* and *P. boydii* being able to penetrate the turtle egg, while the production of proteases and amylases would also allow utilisation of the embryonic tissue as a nutrient source.

7.3.2 PROCEDURE

Enzymic activity of the strains of *F. oxysporum*, *F. solani* and *P. boydii* isolated from failed green turtle eggs at Heron Island was determined according to

the procedures of Hankin and Anagnostakis (1975) and Kunert *et al.* (1993). All tests were conducted by central inoculation onto media designed to detect amylase, lipase, protease, collagenase and elastinase. In addition, the production of organic acids was detected by growth on PDA media containing $0.018gL^{-1}$ of the pH indicator Phenol Red (as recommended by Power and McCuen, 1988). Eight replicates of each test per fungus were incubated at room temperature (21-23°C) until colonies were 5-10mm in diameter.

To detect fungi in embryonic tissue, large embryos beyond Stage 26 (after Miller 1985) were surface sterilised by immersion in 70% alcohol for 5 mins. The heart, liver, pectoral muscle, and small intestine were then removed and each organ placed on half-strength Potato Dextrose Agar with $0.05g\ L^{-1}$ chloramphenicol. Once it had been established that fungi could be cultured from all of these tissues (see Section 7.3.3), only the liver (being the largest organ) was further sampled.

7.3.3 RESULTS

The lytic effects of the fungi on each medium are summarised in Table 7.3.1 as a positive (+) or negative (-) lytic behaviour. All of the replicates for each fungus on each media showed the same response.

Table 7.3.1 Lytic capability of *F. solani, F. oxysporum* and *P. boydii.*

	Fungi		
Metabolites	*F. oxysporum*	*F. solani*	*P. boydii*
Amylase	+	+	+
Collagenase	+	+	+
Elastinase	+	+	+
Lipase	+	+	+
Organic Acids	+	+	+
Protease	+	+	-

The species of fungi cultured from embryonic tissue are reported in Table 7.3.2. In addition to sampling of the embryonic tissue, some eggs also had swabs taken of the fungi on their exterior (see Chapter 4). These have been included in Table 7.3.2 to provide a comparison of fungi cultured from the exterior and embryonic tissue of failed eggs.

Fungi were isolated from 65% of the liver samples. Of these cultures, 84.6% were *P. boydii*, 7.7% *F. solani*, and 7.7% a mixed culture of *P. boydii and F. solani*. Swabs taken from the exterior of 7 eggs prior to tissue sampling all produced *P. boydii*. In one egg, *P. boydii* was isolated from both the exterior and liver samples, and *F. solani* was also present in this tissue. Three other eggs also had mixed cultures isolated from the embryonic tissues.

Table 7.3.2 Fungi isolated from tissues of dead sea turtle embryos.
(- indicates nil sample taken)

		Tissue Isolate				
Specimen	Turtle	Liver	Heart	Muscle	Gut	Egg Surface
1	Green	*P. boydii*	*P. boydii*	*F. solani*	*P. boydii*	-
2	Green	*F. solani*	-	*P. boydii*	*F. solani*	-
3	Green	Nil growth	*P. boydii*	Nil growth	*P. boydii*	*P. boydii*
4	Green	Nil growth	Nil growth	Nil growth	Nil growth	*P. boydii*
5	Green	*P. boydii*	Nil growth	*F. solani*	*F. solani*	-
26	Green	Nil growth	-	-	-	*P. boydii*
29	Green	Nil growth	-	-	-	*P. boydii*
31	Green	*P. boydii*	-	-	-	-
32	Green	Nil growth	-	-	-	*P. boydii*
33	Green	Nil growth	-	-	-	-
34	Green	*P. boydii*	-	-	-	-
35	Green	*P. boydii*	-	-	-	-
36	Green	*P. boydii*	-	-	-	-
38	Green	*P. boydii*	-	-	-	-
39	Green	*P. boydii*	-	-	-	-
41	Green	*P. boydii*	-	-	-	-
42	Green	*F. solani, P. boydii*	-	-	-	*P. boydii*
44	Green	*P. boydii*	-	-	-	-
45	Green	*P. boydii*	-	-	-	-
46	Loggerhead	Nil growth	-	-	-	*P. boydii*

7.3.4 DISCUSSION

Enzymes and organic acids produced by *F. oxysporum, F. solani* and *P. boydii* (see Table 7.3.1) should allow invasion of the organic component of the sea turtle eggshell and utilisation of the eggshell, albumen and yolk as nutrients. This would ultimately result in embryonic death after disruption of the eggshell integrity, loss of respiratory surface, invasion of the embryo, and/or embryo malnutrition.

The prevalence of *P. boydii* in tissue samples reflects the occurrence of this fungus on the egg exterior relative to other species. Fungi were isolated from only 65% of liver samples, yet the embryos were selected from eggs with obvious fungal growth over the exterior. This may indicate that fungal invasion of embryonic tissue only occurs post-mortem, and the time period between colonisation of the egg and tissue sampling was not sufficient to allow fungal penetration of the eggshell to access the egg contents in all samples. Post-mortem fungal penetration of the eggshell by more than one species seems unlikely in the presence of only a single external colony.

Previous studies typically show monotypic cultures from the egg exterior, with only 6.5% of samples (n=107) revealing mixed cultures (1 *F. oxysporum* + *F. solani*; 2 *F. oxysporum* + *P. boydii*, 4 *F. solani* + *P. boydii* – see Chapter 4). In the event of multiple fungal species penetrating the eggshell, the fungus isolated from the egg exterior may represent the initial invader ("founder") species, or the climax community following competitive exclusion.

In future studies of fungal invasion of reptilian eggs it is suggested any external isolates should not be regarded as the causative agent of embryo mortality until there are comparative cultures of multiple-organ tissue samples. Samples should be taken as close to embryo mortality as possible to prevent possible community succession of microbes. If multiple isolates are obtained from the egg exterior and/or tissue, fungal competition studies could be conducted to determine possible order of invasion.

7.4 THE PRODUCTION OF MYCOTOXINS

Mycotoxins are fungal secondary metabolites, whose biological effects on other organisms include acute and chronic cytotoxicity, neurotoxicity, immunosuppressive activity, teratogenicity, mutagenicity, and carcinogenicity. While there are no published data on the production of mycotoxins by *P. boydii*, the fusaria are known toxigenic fungi (see Table 7.4.1). Lethality to chicken embryos after direct exposure to low dosages (ranging from 0.08μg egg^{-1} of diacetoxyscirpenol to 5.0μg egg^{-1} of noesolaniol– see Betina 1989) suggests mycotoxins may also have a high toxicity for sea turtle eggs.

Mycotoxicosis of mammals occurs after exposure to mould dusts (generated by colony disturbance) via skin contact, ingestion or inhalation (Betina 1989). Sea turtle embryos could be exposed to toxic metabolites if hyphae penetrated the egg and invaded embryonic tissue, or if mycotoxins diffused through the eggshell from fungus on the egg exterior. The survival of fungus-free eggs adjacent to those showing fungal growth on their exterior (see Chapter 6), suggests that, if operant, detrimental influences of fusaria mycotoxins are limited to eggs that have been colonised.

Table 7.4.1 Known mycotoxins of *F. oxysporum* and *F. solani.*

Mycotoxin	Systematic Name	Molecular Formula	Produced by *F. oxysporum*	Produced by *F. solani*
Diacetoxyscirpenol	4β,15-Diacetoxy-12,13-epoxy-trichothec-9-ene-3α,5β,15-triol	$C_{19}H_{26}O_7$	Yes	Yes
Noesolaniol	4β,15-Diacetoxy-12,13-epoxy-trichothec-9-ene-3α,4β,8α,15-tetrol	$C_{19}H_{26}O_8$	No	Yes
HT-2 Toxin	15-Acetotoxy-8-(3-methylbutanoyl)-12,13-epoxy-trichothec-9-ene-3α,4β,8α,15-tetrol	$C_{22}H_{32}O_8$	No	Yes
T-2 Toxin	4β,15-Aiacetoxy-8-(3 methylbutanoyl)-12,13-epoxy-trichothec-9-ene-3α,4β,8α,15-tetrol	$C_{24}H_{34}O_9$	Yes	Yes
Fusarenone	4β-Acetoxy-12,13-epoxy-3α, 7α,15-trihydroxy-trichothec-9-en-8-one	$C_{17}H_{22}O_8$	No	Yes
Zearalenone	-	$C_{17}H_5O_5$	Yes	Yes
Moniliformin	3-Hydroxy-3-cyclobutene-1,2-dione	C_4HO_3Na or C_4HO_3K	Yes	No
Nivalenol	12,13-Apoxy-3α,4β,7α,15-tetra-hydroxy-trich-9-en-8-one	$C_{15}H_{20}O_7$	*Fusarium* spp.	

(adapted from Betina 1989)

7.5 EMBRYONIC MALNUTRITION AFTER FUNGAL COLONISATION OF SEA TURTLE EGGS

Kunert *et al.* (1993) describe mortality of snake (*Elaphe guttata*) eggs in the presence of *F. solani*, which has strong proteolytic and lipolytic activity allowing digestion of the egg yolk. The stunted growth of a single hatchling produced from an affected egg was attributed to yolk coagulation leading to malnutrition and reduction of available space after invasion of the egg compartments by *F. solani*. Hibberd and Harrower (1993) also observed hyphal penetration into air spaces of *C. porosus* eggs, but there were no reports of coagulated yolks in affected eggs (Hibberd and Harrower 1993).

Kunert *et al.* (1993) detected protease and lipase production by *F. solani*, but did not show that this fungus caused the yolk coagulation observed in snake eggs. Coagulation could have been caused by bacterial action or dehydration of the yolk in the absence of embryonic metabolism in non-viable eggs. While *F. solani* and *P. boydii* have been isolated from sea turtle embryonic tissue (see Chapter 7.3), the presence of fungal hyphae and/or spores within the yolk was not investigated histologically or by inoculation onto media. However, excavation of sea turtle nests and inspection of eggs displaying external signs of fungal invasion did not reveal hyphal masses between the eggshell and embryonic membranes, or coagulation of the yolk. Observations during this study do not support the hypothesis that colonisation of eggs by *F. solani* results in embryo malnutrition.

CHAPTER 8

THE INFLUENCE OF THE NEST ENVIRONMENT ON FUNGAL COLONISATION OF SEA TURTLE NESTS AT HERON ISLAND

8.1 INTRODUCTION

Nest site selection on the nesting beach can influence embryo survival, hatchling survival and sex ratio, thereby affecting the fitness of the reproductive population (Mrosovsky and Yntema 1980, Ackerman *et al.* 1985, Whitmore and Dutton 1985, Mrosovsky and Provancha 1989, Mrosovsky 1994, Ackerman 1996). While Mrosovsky (1983), Eckert (1987), Tucker (1990) and Hays *et al.* (1995) believe nest site selection to be a random choice by the female, there is evidence that beach topography (Dodd 1988, Horrocks and Scott 1991, Crain *et al.* 1995, Hays *et al.* 1995, Mortimer 1995), vegetation (Horrocks and Scott 1991, Hays and Speakman 1993, Mortimer 1995), light pollution (Mortimer 1995, Salmon *et al.* 1996), thermal cues (Stoneburner and Richardson 1981), inter-specific competition (Whitmore and Dutton 1985) and human disturbance (William-Walls *et al.* 1983, Witherington 1992) influence nesting behaviour. It is possible that choice of nest site and the subsequent nest environment may also have a direct effect on the likelihood of sea turtle eggs being colonised by fungus.

To investigate possible influences of the nest environment on the fungal invasion of sea turtle nests, parameters of the green turtle nest and nest environment at Heron Is. were investigated since this was the largest data set available. Results were then compared with conditions in sea turtle nests at other rookeries in eastern Australia.

8.2 PROCEDURE

8.2.1 The Influence of Nest Characteristics on Fungal Colonisation of Green Turtle Nests at Heron Is.

Analyses were carried out on data from post-emergence excavations of green turtle nests at Heron Island in the 1996/97 and 1997/98 nesting seasons (pooled data). The following nest parameters were investigated for their potential influence on hatch success, the proportion of failed eggs colonised by fungi, and the proportion of the clutch colonised by fungi.

Nest Habitat was recorded categorically as the nest position with regard to elevation (B- beach, below the front slope of the dune; S- slope, front slope of the dune; D- dune) and vegetation (S- bare sand area, no vegetation; G- grass area; SH- nest beneath shrub; T- nest beneath tree). At Heron Is., the usual nest habitats are BS, SS, ST, DG, DS, DSH and DT.

Nest Depth was the measured distance from the bottom of the excavated nest to the natural beach surface, rounded to the nearest centimetre.

Clutch Size was estimated from the number of eggshells + unhatched eggs + undeveloped eggs (see Chapter 2).

The Number of Dead Eggs Within the Clutch was the total of unhatched eggs + undeveloped eggs. Predated eggs were not included in this category as they had usually lost their contents and thus would not provide a nutrient source for fungi.

Sand samples were also collected during the 1997/98 nesting season from amongst the nest contents into sterile bottles and stored out of direct sunlight at room temperature until analyses. The following substrate characteristics were measured following procedures by Tan (1996) unless noted otherwise.

Substrate pH was measured by taking a 5g sample of sand that had been oven dried to constant weight at 65°C in a Qualtex Solidstat Incubator, mixing it with 5mL of deionised water and shaking at 40rpm for 15mins on a Lab-Line Junior Orbit Shaker. The substrate pH was determined by the Potentiometric Method using a T.P.S. LC80 pH-mV Meter. The electrode was calibrated against pH 7.0 and pH 4.0 buffer solutions prior to use, rinsed with distilled water between samples and re-calibrated every 20 samples.

Substrate Conductivity was measured by taking a 5g sample of dried sand, mixing it with 5mL of deionised water and shaking at 40rpm for 15mins on a Lab-Line Junior Orbit Shaker. The substrate conductivity was measured with a TPS LC84 Digital Salinity-Conductivity Meter as mS cm^{-1}.

Substrate Moisture Content was measured by the gravimetric method as a direct determination of water content. A 5g sample of sand was dried in a Qualtex Solidstat Incubator at 65°C until there was no further change in sample weight and the weight loss recorded. The wet mass percentage water content was calculated as (wet weight-dry weight)/wet weight x 100.

Substrate Organic Content was determined using porcelain crucibles that had been washed in 5% hydrochloric acid for 24hrs, rinsed with water and then fired at 500°C

in a muffle furnace for 5hrs to remove all moisture. A 2g sample of oven dried sand was fired in the crucibles at 500°C for 5hrs to combust organic matter. The percentage organic content was calculated as: weight loss/wt dry sand x 100.

Substrate Mycobiota Density: a 2g sample of sand, that had been oven dried to constant weight at 30°C to avoid killing any fungi, was mixed with 18mL of sterile distilled water to give a 10^{-1} dilution and shaken at 40rpm for 30mins on a Lab-Line Junior Orbit Shaker. Care was taken not to overly agitate the soil-water solution as this can rupture mycelia and sporing bodies into numerous fragments, each capable of producing a single colony (Warcup 1955). After shaking, 2mL of the mixture was pipetted into 18mL of sterile distilled water to give a 10^{-2} dilution. This dilution was chosen to produce plates that had plate counts within the range of 20-300 as recommended by Tate (1995). A 1mL aliquot of this solution was then pippetted into the base of a sterile agar plate and mixed with 15mL of sterile, molten PDA with 0.05g L^{-1} chloramphenicol included to inhibit bacterial growth. Three plates of each dilution of each sample were incubated at 28°C for 48hrs, after which the number of colonies on each plate were counted. The number of viable fungal units per gram of dry soil (vfu g^{-1}) for each sample was calculated by multiplying the mean plate count by the dilution factor and dividing by the dry weight of the sand sample.

Nest, clutch and substrate characteristics were analysed by Chi-square tests for heterogeneity (where data were categorical) or linear regression (where data were discrete or continuous). Chi square comparisons were conducted for:

a) the number of hatched eggs and the number of unhatched eggs

b) the number of failed eggs colonised by fungi and number of failed eggs

without signs of fungal growth

and, c) the number of eggs colonised by fungi and number of eggs without signs of fungal growth

Where regression analyses were used, percentage hatch, percentage failed eggs colonised by fungi and percentage of the total clutch colonised by fungi were separately regressed on each of the nest characteristics. Since the number of dead eggs is a direct representation of hatch success, these data were not analysed.

8.2.2 The Nest Environment of Sea Turtle Rookeries in Eastern Australia

Variation in nest characteristics among green and loggerhead turtle rookeries was analysed using one-way analyses of variance, with *post-hoc* Tukey tests applied where appropriate. Since only a single rookery was investigated each for flatback and hawksbill turtles, they were not included in analyses.

8.3 RESULTS

8.3.1 The Influence of the Nest Environment on Fungal Colonisation of Green Turtle Nests at Heron Is.

Factors of the nest environment contributing to significant variation in hatch success and fungal invasion of sea turtle nests at Heron Is. are summarised in Table 8.1 and Figures 8.1-8.24. Data used in the Chi-square analysis for variation in egg failure and colonisation by fungi with nest habitat are given in Table 8.2, with factors that deviate significantly from the overall expected ratios highlighted in bold.

Table 8.1 Nest characteristics contributing to variation in the hatch success and fungal colonisation of green turtle nests at Heron Is. (significant factors are in bold). Regression equations are not given for non-significant factors.

Factor of the Nest Environment	Statistical Test	Hatch Success	Proportion of Failed Eggs with Fungi	Proportion of Clutch with Fungi
Nest Habitat	Chi-square	**χ^2_6=146.35, *P*<0.0001**	**χ^2_6=69.32, *P*<0.0001**	**χ^2_6=46.74, *P*<0.0001**
Nest Depth	Regression	**r^2=0.033, F_{191}=6.473, *P*=0.012 y=78.061 + 0.155*x***	F_{191}=0.147, *P*=0.701	F_{191}=1.671, *P*=0.198
Clutch Size	Regression	F_{192}=0.757, *P*=0.385	F_{192}=1.662, *P*=0.199	F_{192}=1.662, *P*=0.199
Number of Dead Eggs	Regression	Direct representation	**r^2=0.061, F_{192}=12.407, *P*=0.001 y=68.710+0.781*x***	**r^2=0.852 F_{192}=1109.000, *P*<0.001 y=-0.503+0.911x**
Substrate Moisture	Regression	F_{49}=0.019, *P*=0.890	F_{49}=1.713, *P*=0.197	F_{49}=0.000, *P*=0.992
Substrate Organic	Regression	F_{49}=0.002, *P*=0.967	F_{49}=0.133, *P*=0.717	F_{49}=0.031, *P*=0.862
Substrate pH	Regression	F_{49}=0.445, *P*=0.508	F_{49}=0.012, *P*=0.914	F_{49}=0.216, *P*=0.644
Substrate Conductivity	Regression	**r^2=0.219, F_{49}=13.719, *P*=0.001 y=101.047-7.423*x***	F_{49}=0.011, *P*=0.916	**r^2=0.180, F_{49}=10.787, *P*=0.002 y=-1.631+6.728x**
Substrate Mycobiota	Regression	F_{48}=0.000, *P*=0.999	F_{48}=0.448, *P*=0.506	F_{48}=0.189, *P*=0.665

Figure 8.1 Variation in Failure and Fungal Colonisation of Eggs (Mean±SD) with Habitat of Green Turtle Nests at Heron Is.

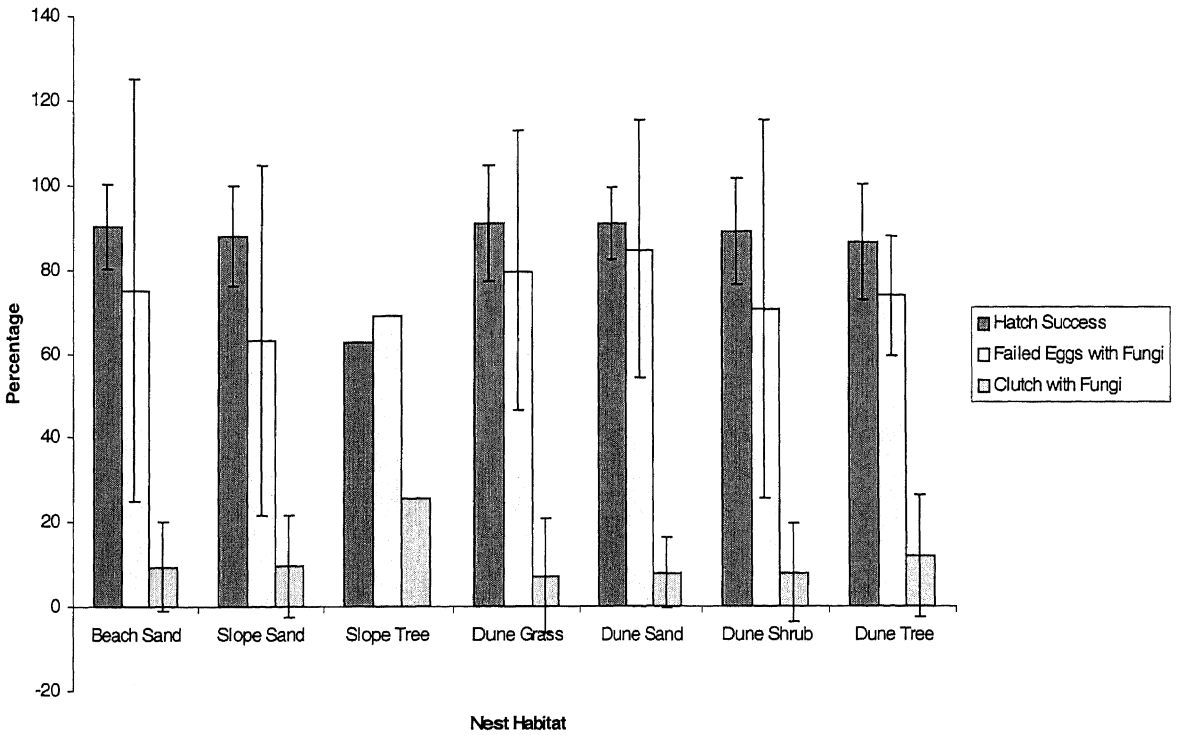


Figure 8.2 Variation in Hatch Success with Depth of Green Turtle Nests at Heron Is. (P =0.012, y=78.061+0.155x).

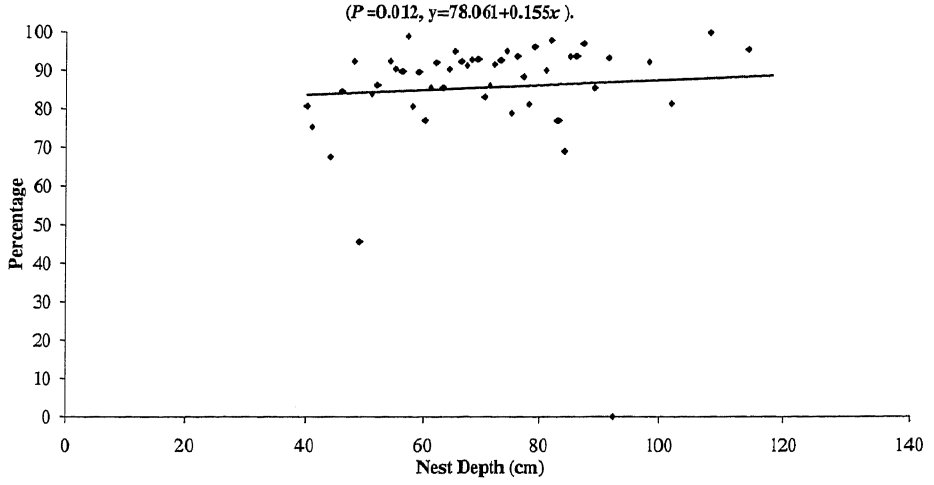


Figure 8.3 Variation in the Percentage of Failed Eggs Colonised by Fungi with Depth of Green Turtle Nests at Heron Is. (P =0.701).

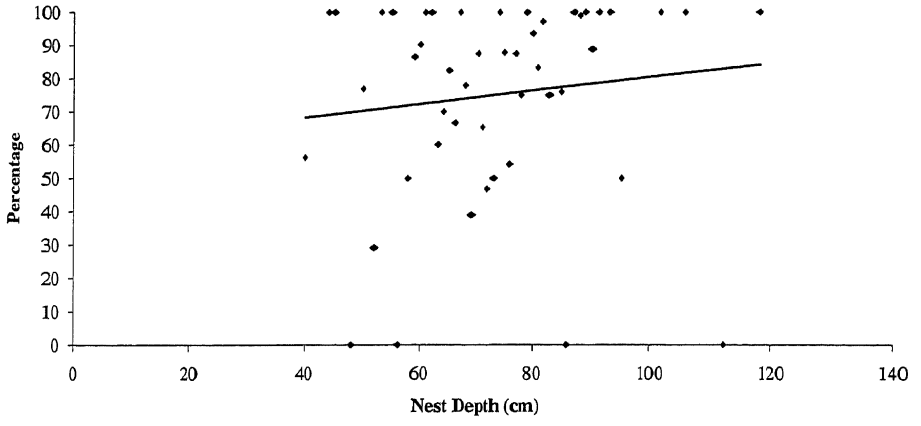


Figure 8.4 Variation in the Percentage of the Clutch Colonised by Fungi with Depth of Green Turtle Nests at Heron Is. (P =0.198).

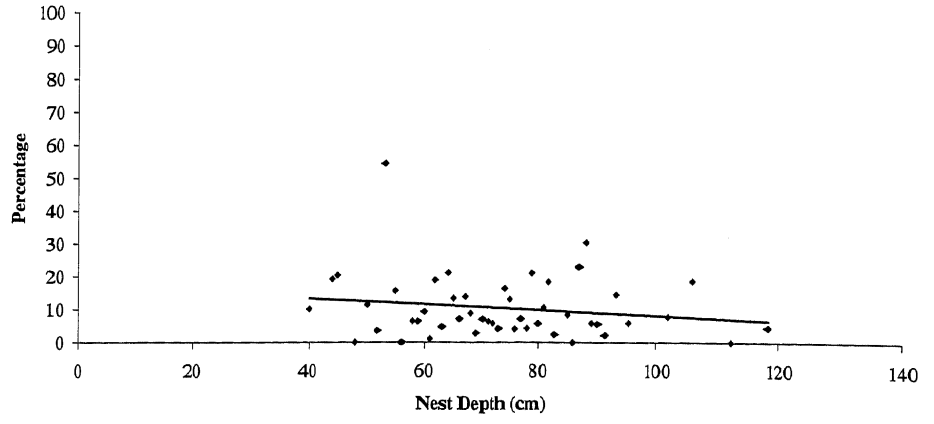


Figure 8.5 Variation in Hatch Success with Clutch Size of Green Turtle Nests at Heron Is. (P=0.385).

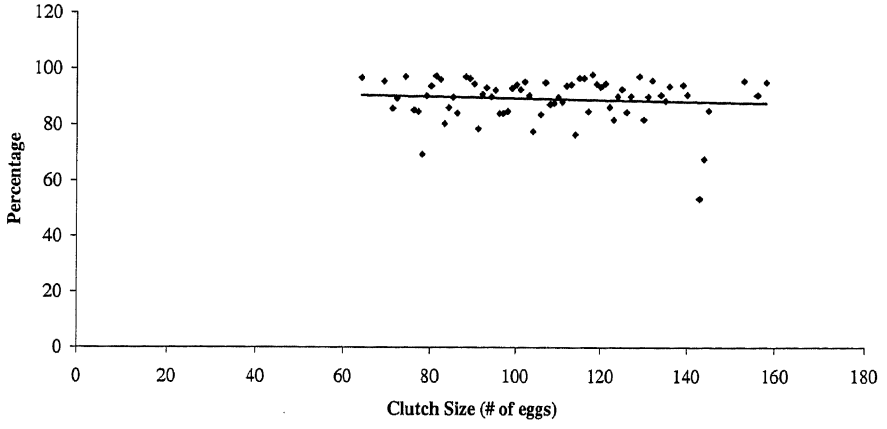


Figure 8.6 Variation in the Percentage of Failed Eggs Colonised by Fungi with Clutch Size of Green Turtle Nests at Heron Is. (P=0.199).

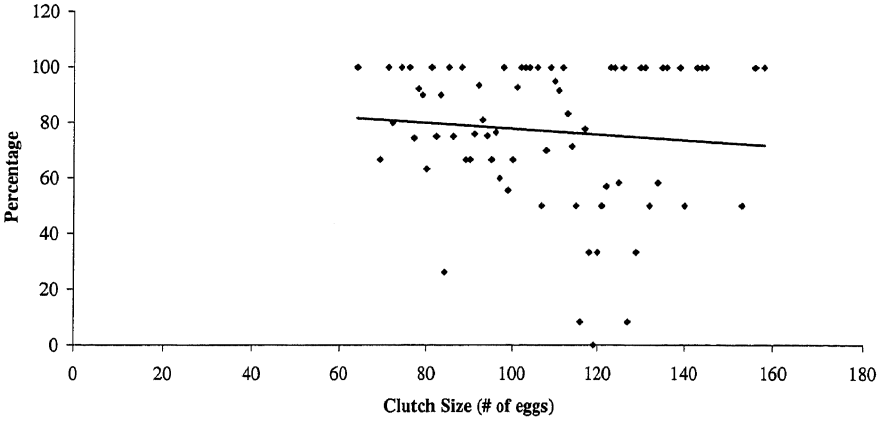


Figure 8.7 Variation in the Percentage of the Clutch Colonised by Fungi with Clutch Size of Green Turtle Nests at Heron Is. (P = 0.199).

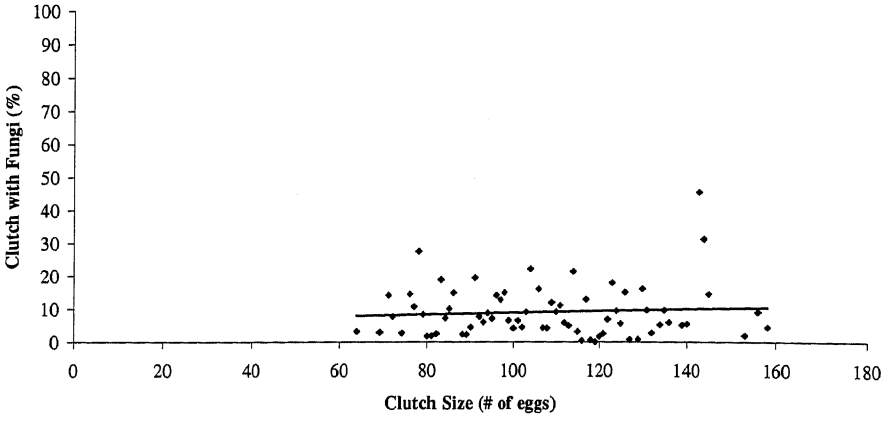


Figure 8.8 Variation in the Percentage of Failed Eggs Colonised by Fungi with the Number of Dead Eggs in Green Turtle Nests at Heron Is. (P =0.001, y=0.503+0.911x).

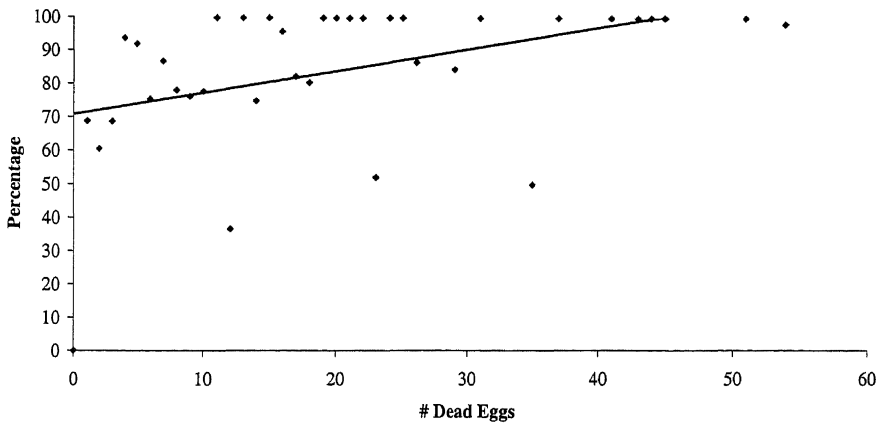


Figure 8.9 Variation in the Percentage of the Clutch Colonised by Fungi with the Number of Dead Eggs In Green Turtle Nests at Heron Is. (P <0.001, y=-0.503+0.911x).

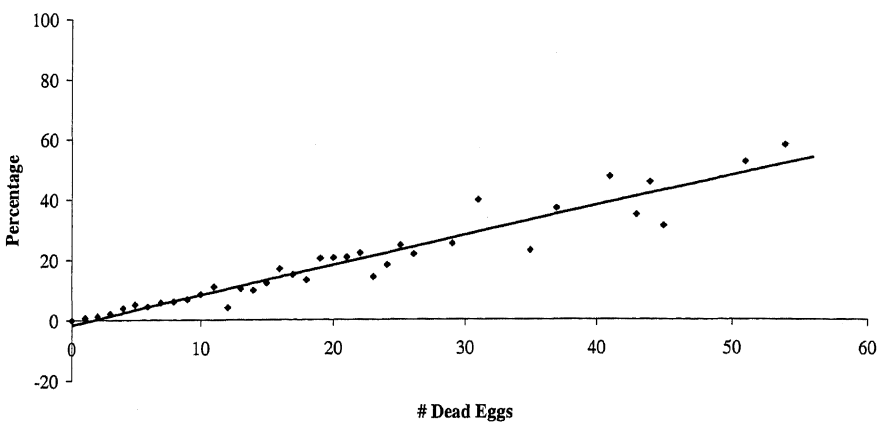


Figure 8.10 Variation in Hatch Success with Substrate Moisture Content of Green Turtle Nests at Heron Is. (P =0.890).

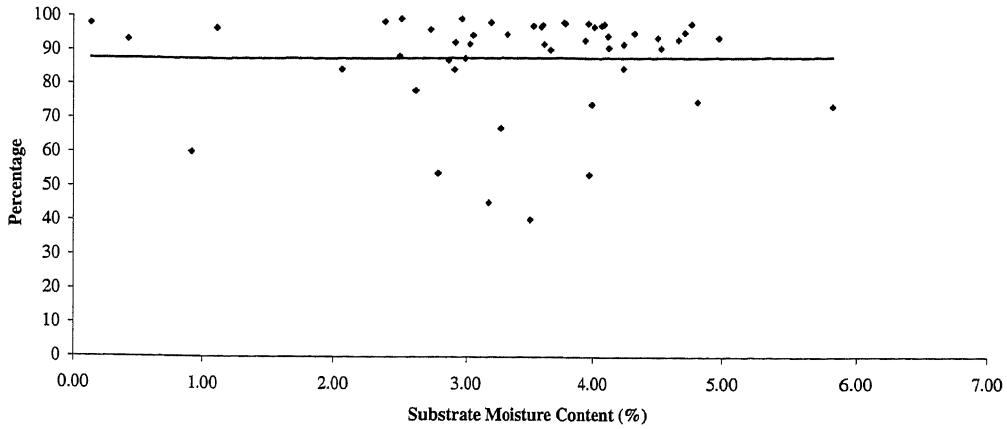


Figure 8.11 Variation in the Percentage of Failed Eggs Colonised by Fungi with Substrate Moisture Content of Green Turtle Nests at Heron Is. (P =0.197).

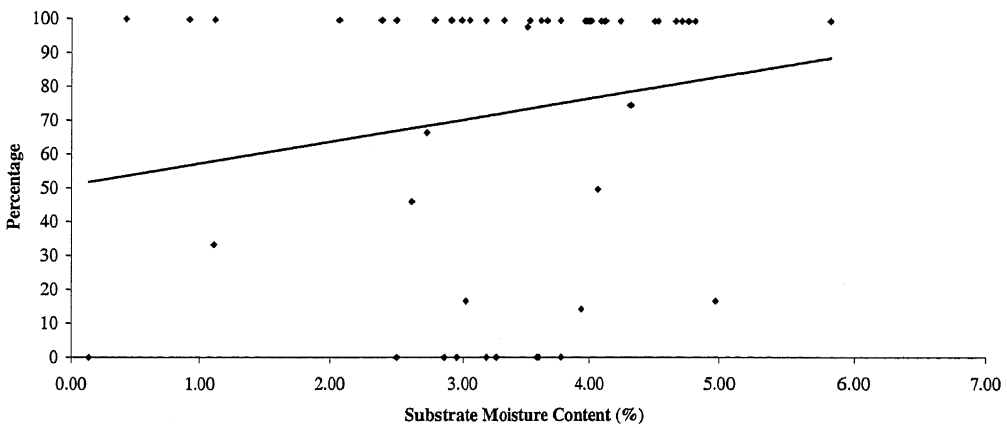


Figure 8.12 Variation in the Percentage of the Clutch Colonised by Fungi with Substrate Moisture Content of Green Turtle Nests at Heron Is. (P =0.992).

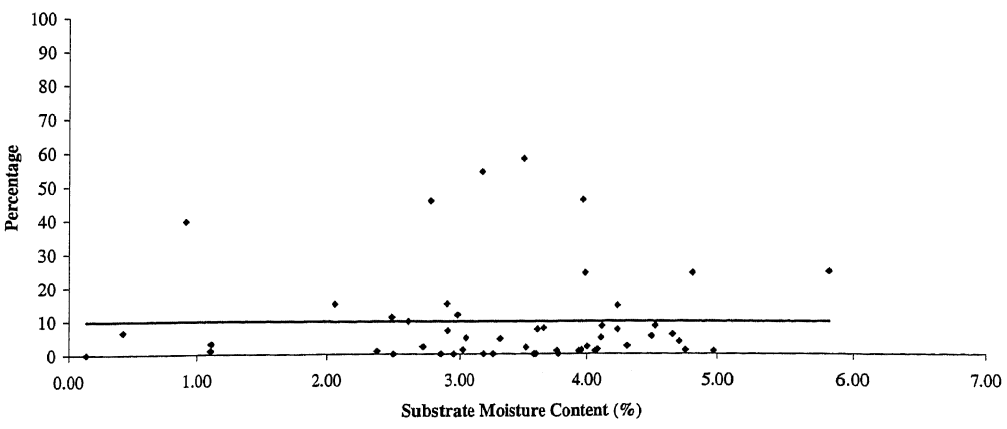


Figure 8.13 Variation in Hatch Success with Substrate Organic Content of Green Turtle Nests at Heron Is. (P =0.967).

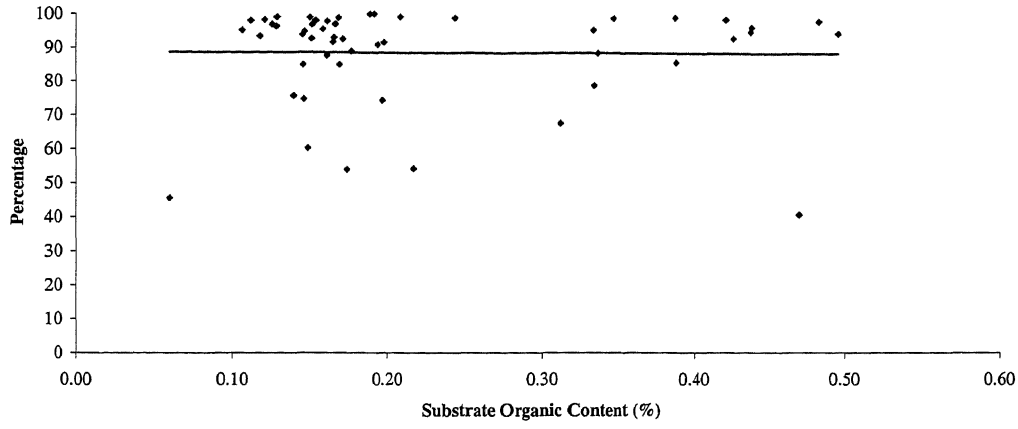


Figure 8.14 Variation in the Percentage of Failed Eggs Colonised by Fungi with Substrate Organic Content of Green Turtle Nests at Heron Is. (P =0.717).

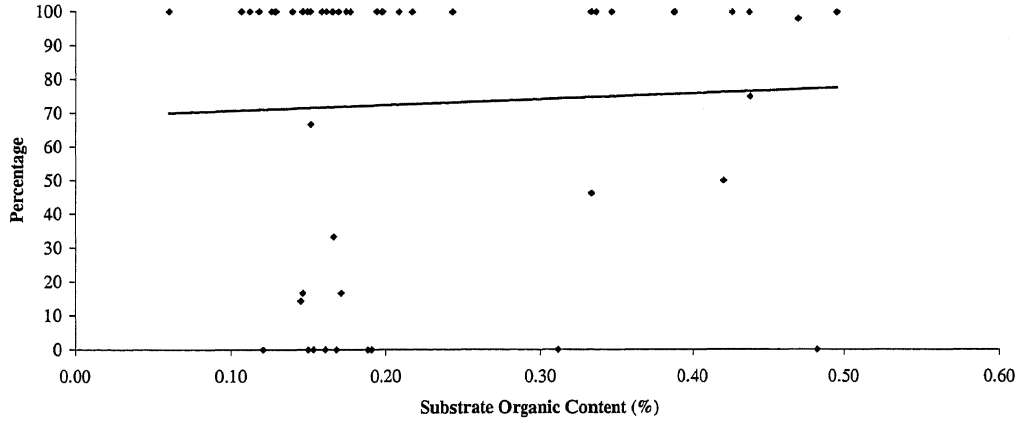


Figure 8.15 Variation in the Percentage of the Clutch Colonised by Fungi with Substrate Organic Content of Green Turtle Nests at Heron Is. (P =0.862).

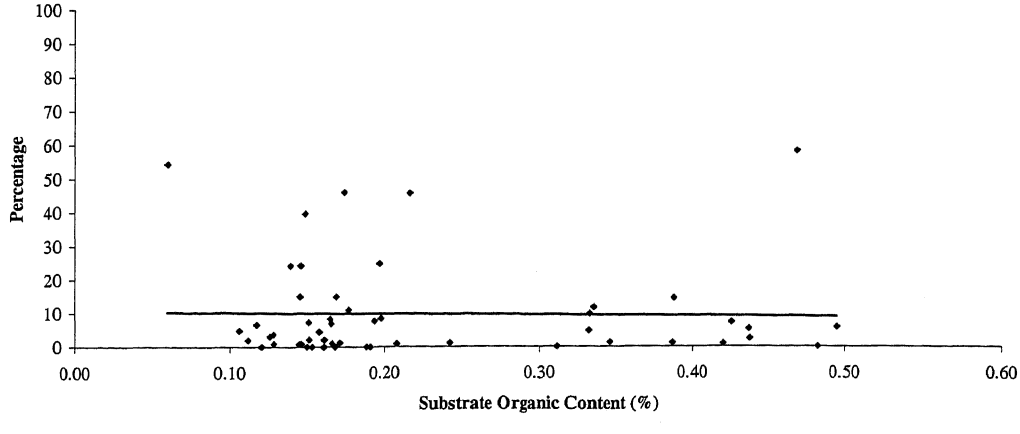


Figure 8.16 Variation in Hatch Success with Substrate pH of Green Turtle Nests at Heron Is. (P =0.508).

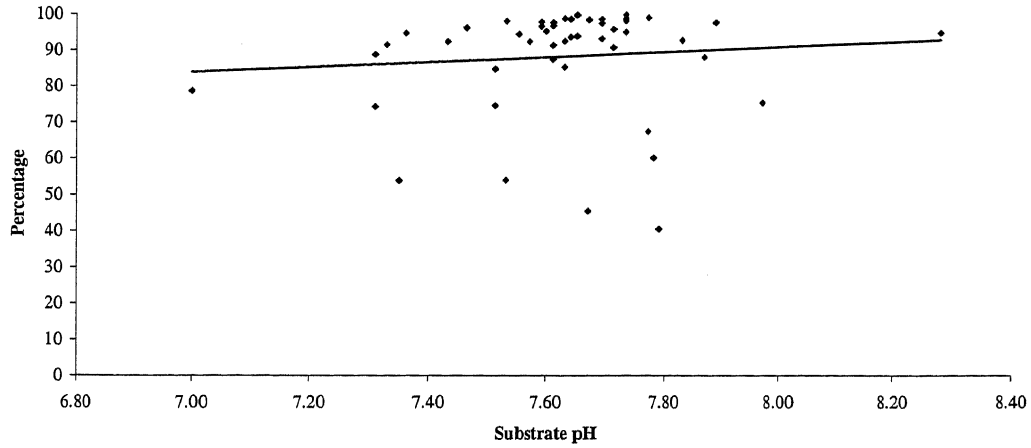


Figure 8.17 Variation in the Percentage of Failed Eggs Colonised by Fungi with Substrate pH of Green Turtle Nests at Heron Is. (P =0.508).

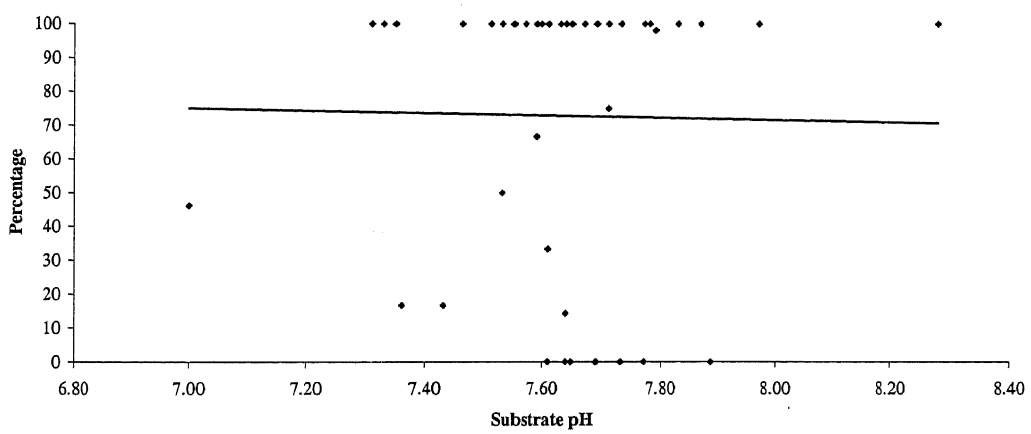


Figure 8.18 Variation in the Percentage of the Clutch Colonised by Fungi with Substrate pH of Green Turtle Nests at Heron Is. (P =0.445).

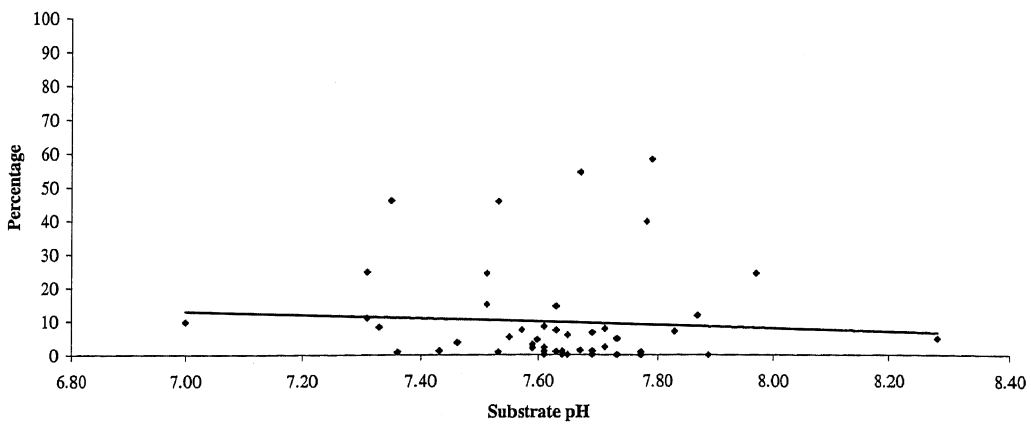


Figure 8.19 Variation in Hatch Success with Substrate Conductivity of Green Turtle Nests at Heron Is. (P=0.001, y=101.047-7.423x).

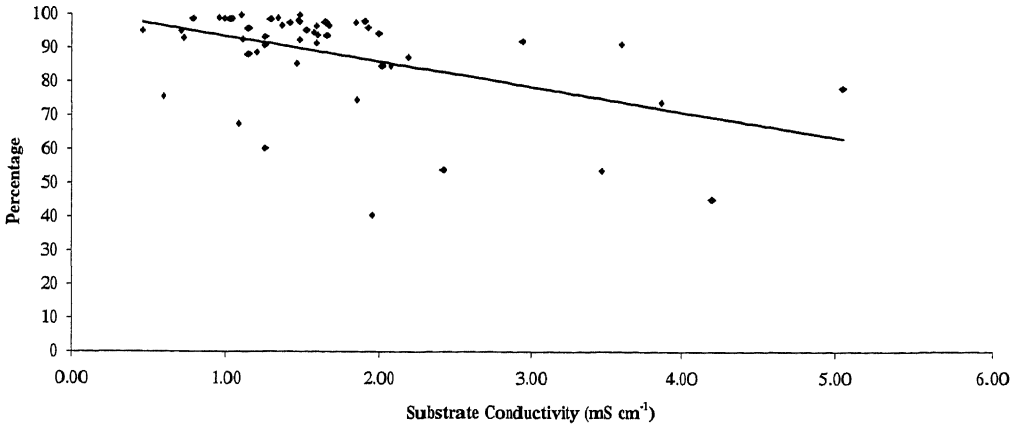


Figure 8.20 Variation in the Percentage of Failed Eggs Colonised by Fungi with Substrate Conductivity of Green Turtle Nests at Heron Is. (P=0.916).

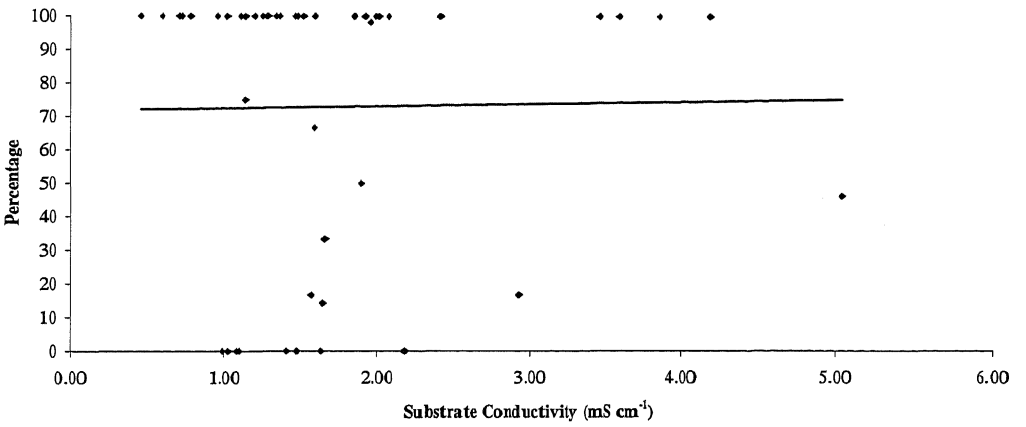


Figure 8.21 Variation in the Percentage of the Clutch Colonised by Fungi with Substrate Conductivity of Green Turtle Nests at Heron Is. (P=0.002, y=-1.631+6.728x).

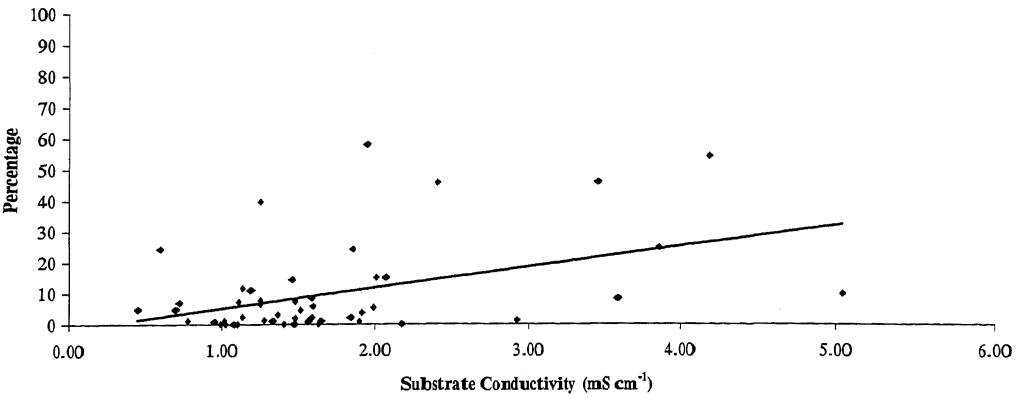


Figure 8.22 Variation in Hatch Success with Substrate Mycobiota Density of Green Turtle Nests at Heron Is. (P=0.999).

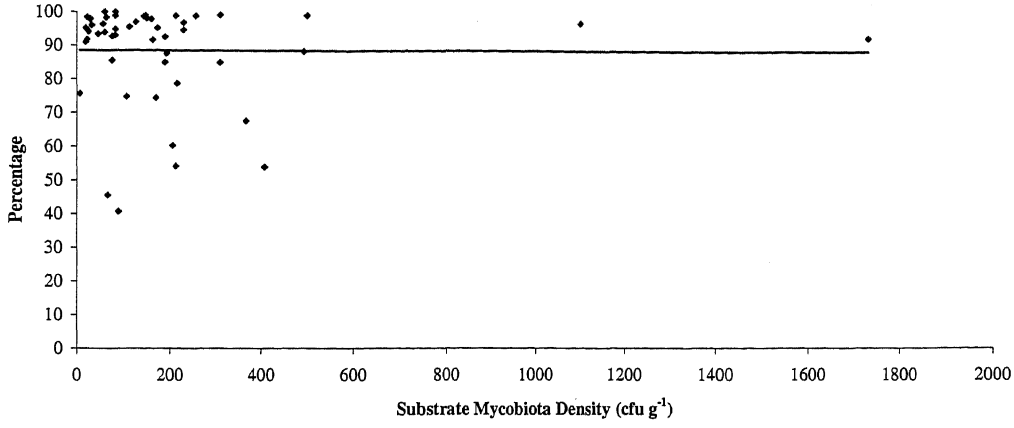


Figure 8.23 Variation in the Percentage of Failed Eggs Colonised by Fungi with Substrate Mycobiota Density of Green Turtle Nests at Heron Is. (P=0.506).

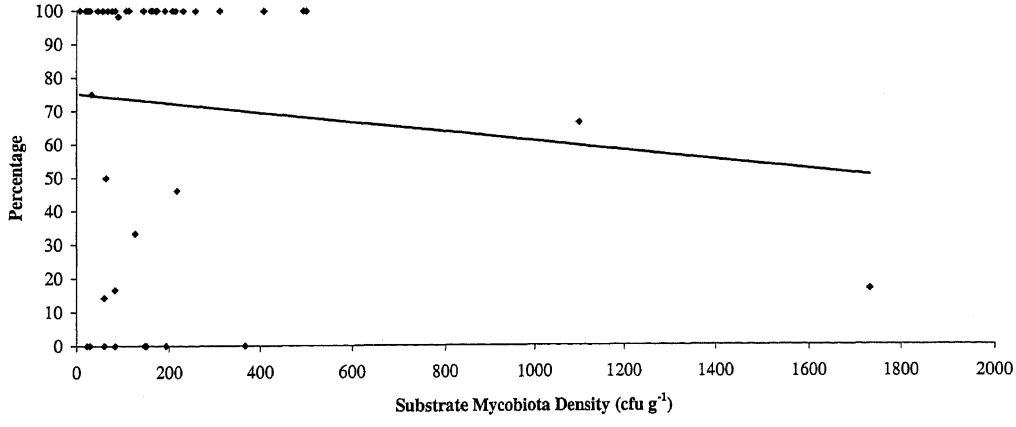


Figure 8.24 Variation in the Percentage of the Clutch Colonised by Fungi with Substrate Mycobiota Density of Green Turtle Nests at Heron Is. (P=0.665)

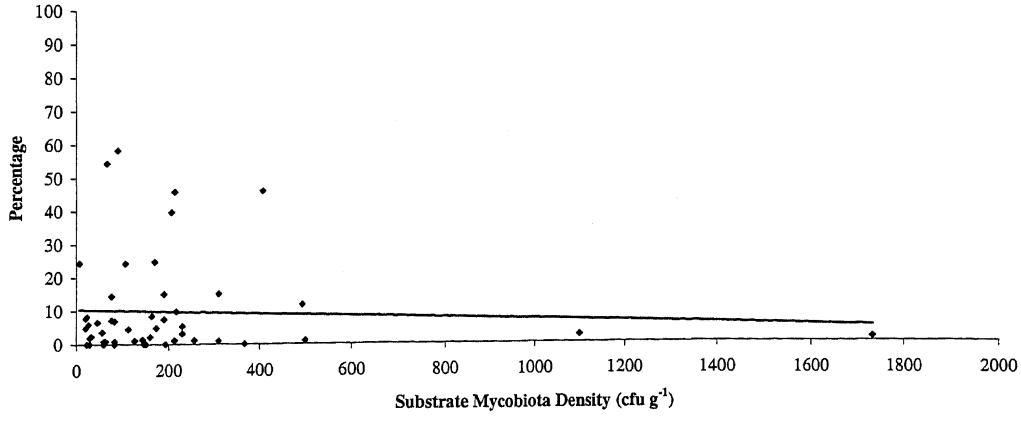


Table 8.2 Egg counts for 3 separate Chi-square analyses to determine variation in green turtle egg failure and colonisation by fungi with nest habitat at Heron Is.. Factors significantly deviating from the expected mean are given in bold.

		Number					
Habitat	Nests examined	Hatched Eggs	Failed Eggs	Failed Eggs with Fungi	Failed Eggs without Fungi	Eggs with No Fungi	Eggs with Fungi
BS	4	363	34	32	2	365	32
SS	23	2162	284	**233**	**51**	**2214**	**233**
ST	1	**49**	**29**	**20**	**9**	**58**	**20**
DG	11	**959**	**88**	71	17	976	71
DS	82	**7703**	**720**	**668**	**52**	7763	668
DSH	19	1672	218	**163**	**55**	1729	163
DT	54	**4748**	**723**	635	88	4836	635

Hatch success of green turtle nests varied significantly with nest habitat, nest depth and substrate conductivity. Nest habitat and the number of dead eggs in the clutch significantly affected the percentage of failed eggs in the clutch invaded by fungus. The percentage of the clutch invaded by fungus varied significantly with nest habitat, the number of dead eggs in the clutch and substrate conductivity.

8.3.2 The Nest Environment of Sea Turtle Rookeries in Eastern Australia

Results of nest excavations and substrate analyses to determine characteristics of the nest environment in eastern Australian turtle rookeries are summarised in Tables 8.3 and 8.4. Results of one-way analyses of variance comparing nest characteristics among green and loggerhead turtle rookeries of eastern Australia to determine significant factors are given in Table 8.5 and 8.6. Significant rookery subgroups, as determined by *a posteriori* Tukey tests, are indicated.

Table 8.3 Nesting habitats of sea turtles in eastern Australia

Rookery	Species	Total # Nests	Nest Habitat (% Occurrence) BS	SS	ST	DG	DS	DSH	DT
Heron Is.	Green	194	2.1	11.9	0.5	5.7	42.3	9.8	27.8
	Loggerhead	7	28.6	14.3	0.0	0.0	28.6	0.0	28.6
Milman Is.	Green	7	0.0	0.0	0.0	0.0	28.6	0.0	71.4
	Hawksbill	32	0.0	0.0	0.0	9.4	21.9	21.9	46.9
Mon Repos	Loggerhead	12	*	16.7	*	0.0	83.3	*	*
Peak Is.	Flatback	44	0.0	20.5	-	0.0	79.6	-	-
Wreck Is.	Green	39	2.6	2.6	0.0	2.6	71.8	15.4	5.1
	Loggerhead	7	0.0	0.0	0.0	0.0	85.7	0.0	14.3

*Nests commonly relocated from this habitat
- Habitat not available at this rookery

Table 8.4 Characteristics of sea turtle nests in eastern Australia. Values given are Mean±SD (number of nests examined)

Rookery	Turtle	Nest Depth (cm)	Clutch Size	# Failed Eggs	Substrate Moisture (%)	Substrate Organic (%)	Substrate pH	Substrate Conductivity (mS/cm)	# Substrate Mycobiota (vfu g^{-1})
Heron Is.	Green	70.8±13.4 (197)	102.0±18.9 (197)	10.8±11.7 (197)	3.37±1.16 (51)	0.22±0.12 (51)	7.36±0.19 (51)	1.70±0.92 (51)	199.1±284.6 (50)
	Loggerhead	48.4±8.9 (7)	108.3±31.5 (7)	38.6±15.6 (7)	3.85±1.97 (5)	0.41±0.22 (5)	7.93±0.44 (5)	2.81±2.32 (5)	69.7±64.2 (5)
Milman Is.	Green	64.00±6.5 (7)	95.6±13.5 (7)	9.1±5.5 (7)	2.50±1.51 (5)	0.22±0.04 (5)	7.65±0.18 (4)	0.36±0.18 (4)	204.7±127.4 (5)
	Hawksbill	47.4±6.9 (32)	114.3±13.5 (32)	13.9±5.5 (32)	1.84±1.12 (10)	0.21±0.04 (10)	7.62±0.58 (8)	0.34±0.27 (7)	313.0±345.7 (9)
Mon Repos	Loggerhead	53.6±9.5 (12)	122.2±17.1 (12)	11.3±6.6 (12)	0.39±0.33 (11)	0.09±0.02 (11)	7.91±0.41 (11)	0.83±0.49 (11)	27.3±38.1 (11)
Peak Is.	Flatback	53.3±6.7 (44)	57.7±8.6 (44)	10.4±9.4 (44)	0.73±0.88 (34)	0.16±0.14 (34)	7.55±0.17 (34)	0.58±0.20 (34)	75.9±51.9 (34)
Wreck Is.	Green	74.2±11.5 (39)	111.2±19.3 (39)	12.4±12.9 (39)	3.11±1.22 (22)	0.26±0.11 (22)	7.60±0.24 (22)	1.08±0.83 (22)	118.1±150.2 (22)
	Loggerhead	58.4±13.3 (7)	117.0±12.3 (7)	28.9±20.8 (7)	2.58±1.69 (6)	0.25±0.03 (6)	7.53±0.41 (6)	0.94±0.46 (6)	80.0±36.9 (6)

Table 8.5 Results of one-way analyses of variance for the influence of rookery on intra-specific variation in characteristics of green sea turtle nests. Significant subsets, as determined by *a posteriori* Tukey tests, are indicated for significant factors.

		Subsets	
Nest Characteristic	*F* value	1 (Lowest)	2 (Highest)
Nest Depth	F=2.218, P=0.111		
Clutch Size	F=4.519, P=0.012	Milman Is., Heron Is.	Heron Is., Wreck Is.
Number of Failed Eggs	F=0.394, P=0.674		
Substrate Moisture	F=1.370, P=0.260		
Substrate Organic	F=0.677, P=0.511		
Substrate pH	F=0.204, P=0.816		
Substrate Conductivity	F=7.055, P=0.002	Milman Is., Wreck Is.	Wreck Is., Heron Is.
Substrate Mycobiota	F=0.858, P=0.428		

Table 8.6 Results of one-way analyses of variance for the influence of rookery on intra-specific variation in characteristics of loggerhead sea turtle nests. Significant subsets, as determined by *a posteriori* Tukey tests, are indicated for significant factors.

		Subset		
Nest Characteristic	*F* value	1 (Lowest)	2	3 (Highest)
Nest Depth	F=1.592, P=0.225			
Clutch Size	F=0.973, P=0.393			
Number of Failed Eggs	F=9.033, P=0.001	Mon Repos	Wreck Is., Heron Is.	
Substrate Moisture	F=14.253, P<0.001	Mon Repos	Wreck Is., Heron Is.	
Substrate Organic	F=17.590, P<0.001	Mon Repos	Wreck Is.	Heron Is.
Substrate pH	F=1.969, P=0.167			
Substrate Conductivity	F=5.541, P=0.013	Mon Repos, Wreck Is.	Wreck Is., Heron Is.	
Substrate Mycobiota	F=3.260, P=0.061			

Clutch size and substrate conductivity varied significantly among green turtle rookeries. The number of failed eggs, substrate moisture, substrate organic content and substrate conductivity varied significantly among loggerhead turtle rookeries. Although hawksbill turtle nests at Milman Is. and flatback turtle nests at Peak Is. were not included in the analyses, parameters of their nest are within the ranges of those measured in green and loggerhead turtle nests at other rookeries.

8.4 DISCUSSION

8.4.1 The Influence of Nest Characteristics on Fungal Colonisation of Green Turtle Nests at Heron Is.

The aim of this study was to determine which environmental factors may influence fungal invasion of the sea turtle nest. Even though there was significant variation in hatch success and fungal invasion of eggs with nest habitat of green turtle nests at Heron Is., in most cases this variation was within a relatively narrow range in all habitats (see Figure 8.1), and other factors of the nest environment appeared to have a greater effect.

The percentage of the green turtle clutch invaded by fungi at Heron Is. increased significantly with the number of dead eggs present. Since failed eggs may act as nutrient sources for fungus as it invades adjacent, viable eggs (see Chapter 6), multiple foci would allow a greater proportion of the clutch to be contacted by hyphae.

Therefore, any characteristic of the nest environment contributing to egg failure is likely to indirectly increase the possibility of a nest being invaded by

fungus. Relatively high substrate conductivity significantly decreased hatch success, but the percentage of failed eggs invaded by fungi did not vary with substrate conductivity. This suggests that the range of conductivities that affected embryo mortality did not have an effect on fungal growth. The percentage of the clutch invaded by fungi did vary significantly with substrate conductivity, which can be explained by fungi being able to invade the high percentage of failed eggs.

8.4.2 The Nest Environment of Sea Turtle Rookeries in Eastern Australia

Although several nest characteristics varied significantly among green (clutch size and substrate conductivity) and loggerhead (the number of failed eggs, substrate moisture, substrate organic content and substrate conductivity) turtle rookeries, only substrate conductivity was found to significantly contribute to increased egg failure (Section 8.3.1).

There was significant variation between the substrate conductivity of Heron Is./Wreck Is. and Milman Is. and Mon Repos. Conditions within flatback turtle nests at Peak Is. and hawksbill turtle nests at Milman Is. were within the ranges determined for green turtle nests at Milman Is. and loggerhead turtle nests at Mon Repos, and less than those at Heron Is. and Wreck Is.. High substrate conductivity is probably due to elevated levels of sea spray on the exposed, high wave action coral cays combined with their relatively low rainfall (November-February average rainfall: Heron Is. 424mm; Milman Is. 858mm (as Bamaga December to February only); Mon Repos (as Bundaberg) 595mm; Peak Island (as Rockhampton) 470mm-Bureau of Meteorology, Monthly Rainfall Bulletin). As a result there is probably a high substrate salt concentration, measured as high conductivity. Consequently, the

osmotic potential of the sand at Heron Is. and Wreck Is. would be expected to be very high, with a correspondingly low soil water potential.

The hydric environment of the sea turtle nest often varies with the thermal micro-climate and there may be interaction between the two. The influence of these factors on fungal invasion of sea turtle eggs is investigated in Chapter 9.

CHAPTER 9

THE INFLUENCE OF THE THERMAL AND HYDRIC NEST MICRO-CLIMATE ON FUNGAL COLONISATION OF SEA TURTLE EGGS

9.1 INTRODUCTION

Sea turtle embryo survival and development is greatly influenced by temperature, moisture availability and levels of respiratory gases during incubation. These variables may also affect fungal germination and growth during the colonisation of sea turtle eggs.

Nest temperatures in eastern Australia range from 25.0°C to 33.0 °C in green turtle nests at Heron Is. (Booth and Astill 2001), 26.0°C to 33.0°C in loggerhead turtle nests at Mon Repos (Maloney *et al.* 1990), 27.1 to 33.7 °C in hawksbill turtle nests at Milman Is. (Loop *et al.* 1995) and 25.5°C to 36.5°C in flatback nests at Peak Is. (Hewavisenthi 1999). Sources of heat that affect nest temperature may be extrinsic (dependent on nest location and habitat) and intrinsic (dependent on the stage of embryo development and resulting metabolic rate). Fungal activity within soil varies with the mean soil temperature, provided harmful extremes are not exceeded (Griffin 1972). Optimal growth of most soil fungi occurs between 25-35°C (Griffin 1972), which is within the range of temperatures typically encountered in sea turtle nests.

The water potential of nesting beaches is reported to range from - 2 to - 320kPa at Ascension Island (Mortimer 1990), - 20 to - 40kPa on the Atlantic coast of Florida (Ackerman *et al.* 1991) and - 150 to - 3000kPa at Peak Is. (Hewavisenthi

1999). The water potential inside the egg is - 900kPa (Ackerman 1991), but the water potential of the eggshell which the fungi first contacts is unknown. Complete inhibition of vegetative growth of most soil fungi does not occur until <-6000kPa, in the absence of other limiting factors, but each species will have a water potential at which growth is optimal (Griffin 1972).

Prange and Ackerman (1974) determined a mean oxygen concentration of 20.63% and mean carbon dioxide concentration of 0.12% in green turtle nests. Germination and linear growth of soil fungi is relatively insensitive to oxygen concentrations of >4% and carbon dioxide concentrations of <10% (Griffin 1972). Therefore, the concentrations of respiratory gases in sea turtle nests are unlikely to limit fungal physiology, and thermal and hydric conditions of the nest are likely to have the greatest influence on fungal growth (Griffin 1972). Furthermore, since sea turtle nests exhibit a range of temperature and moisture regimes on a single nesting beach (see Chapter 8), some are more likely than others to be vulnerable to fungal colonisation. A series of experiments was therefore conducted to determine the influence of the thermal and hydric micro-climate on hatch success, fungal colonisation of failed sea turtle eggs and fungal growth.

9.2 PROCEDURE

9.2.1 Exposure of Experimental Eggs to Varying Hydric and Thermal Conditions

To determine the influence of the thermal and hydric micro-climate on hatch success and fungal colonisation of sea turtle eggs, pairs of eggs were incubated in an orthogonal design of 3 temperature and 3 moisture conditions. During incubation, one of each pair of eggs was inverted to cause embryo mortality and thereby provide

a non-viable egg upon which fungi could grow, since colonisation of non-viable eggs is the initial stage that may lead to colonisation of an egg mass (see Chapter 6). The other egg was a control to determine the influence of temperature and moisture on hatch success.

During the 1999/2000 nesting season, 18 eggs from each of 10 green turtles were collected at Heron Is. and transported to Central Queensland University. Eggs from each clutch were randomly assigned to 9 thermal and hydric treatments with all combinations of 26°C, 28°C and 30°C and optimal (14mL water/100g dried sand), drier than optimal (7mL water/100g dried sand) and wetter than optimal (21mL water/100g dried sand) substrate water content. Optimum hydric conditions were determined by the "pinch method" (Blanck and Sawyer 1981) for air-dried sand that had been collected from a depth of 55cm (average nest depth) from the Heron Is. nesting beach. All of the thermal and hydric regimes used in this experiment were characteristic of natural nest conditions in eastern Australia.

Eggs from each clutch with each treatment were incubated as matched pairs on a 2cm deep substrate in a 16cmx10cmx7cm plastic container covered with plastic film to maintain humidity. Each container and its contents was weighed weekly so moisture loss during incubation could be replaced by sub-surface irrigation as required.

Eggs were placed in the incubation container so that they did not contact each other or the container surfaces, thereby preventing direct transfer of fungi.

When the white-spot had developed to half egg size, one of each pair of eggs was inverted to cause embryo mortality.

Chi-square analyses of three-dimensional and 2x2 contingency tables were used to determine the influence of temperature and moisture on both the hatch success and fungal colonisation of sea turtle eggs. At the end of incubation, swabs were taken from all eggs with visible external fungi for identification following the procedures of Chapter 4.

9.2.2 The Influence of the Thermal and Hydric Environment on the Linear Growth Rate of Nest Mycobiota

To determine the influence of substrate water potential and incubation temperature on the linear growth rate of fungi colonising failed sea turtle eggs, 8 special media were prepared with water potentials ranging from - 173kPa to - 3169kPa following the recipes in Appendix A. This encompassed the known range for turtle nesting beaches and hopefully included the water potential of the egg's exterior surface. Three plates of each water potential medium were centrally inoculated with 0.5cmx0.5cm individual samples of pure *F. oxysporum*, *F. solani* and *P. boydii* cultures that had been incubated on PDA for 7 days at 28°C. The perimeter of the inoculum was outlined in permanent marker on the underside of the petri dish. Triplicates of each fungus on each water potential were then incubated at 26°C, 28°C, 30°C and 32°C in an orthogonal design to determine the influence of thermal and hydric conditions on the linear growth rate of fungi.

As each colony approached the periphery of the plate, its linear growth was measured from the outline of the inoculum to the colony perimeter at 4 equally distributed radii. Time was recorded to the nearest minute in order to accurately calculate linear growth rate. As the thermal and hydric regimes being tested were representatives of the range of conditions likely to occur within the natural sea turtle nest, a Model II, 2-factor analysis of variance was used to determine the effect of temperature and moisture on the linear growth rate of each fungus. *A posteriori* multiple comparison tests to compare the differences between all possible pairs of means are not applied in Model II analyses (Zar 1999) and were not conducted.

9.3 RESULTS

9.3.1 Exposure of Experimental Eggs to Varying Hydric and Thermal Conditions

There was an overall hatch success of 75% of the 89 control eggs (1 of the 90 failed to develop a white spot and was excluded from the analyses). Of the 90 experimental eggs, 4 that continued developing after inversion were not included in further calculations.

The hatch success of control eggs (see Table 9.1) was independent of thermal and hydric conditions during incubation (three dimensional χ^2=15.338, P>0.10, df=12). Fungal colonisation of inverted eggs (see Table 9.2) was also independent of thermal and hydric conditions during incubation (three dimensional χ^2=11.915, P>0.50, df=8). However, all of the expected values in the Chi-square analysis of hatch success were less than 1.0 and all of the expected values in the Chi-square analysis of fungal colonisation of failed eggs were less than 5.0. The resulting Chi-square values for both three-dimensional analyses were, therefore,

likely to be biased (Zar 1999). Chi-square analyses of 2x2 contingency tables, where the thermal and hydric variables were considered separately, showed hatch success of green turtle eggs was marginally affected by temperature (χ^2=5.98, P=0.0502, df=2) and significantly affected by moisture (χ^2=6.42, P=0.0404, df=2). Fungal colonisation of inverted green turtle eggs was significantly affected by temperature (χ^2=6.14, P=0.0464, df=2) but not by moisture (χ^2=2.60, P=0.2719, df=2). However, all expected values in both analyses of fungal colonisation of inverted eggs were less than 5.0 so the chi-square values are still likely to be biased due to the small sample sizes and, therefore, significant results should be treated with caution.

Table 9.1 The number of control green turtle eggs that hatched under varying thermal and hydric incubation regimes.

	Temperature		
Moisture	26°C	28°C	30°C
7mL H_2O/100g sand	4 (n=10)	7 (n=9)	6 (n=10)
14mL H_2O/100g sand	6 (n=10)	10 (n=10)	9 (n=10)
21mL H_2O/100g sand	8 (n=10)	8 (n=10)	9 (n=10)

Table 9.2 The number of inverted green turtle eggs colonised by fungi under varying thermal and hydric incubation regimes.

	Temperature		
Moisture	26°C	28°C	30°C
7mL H_2O/100g sand	9 (n=10)	10 (n=10)	8 (n=10)
14mL H_2O/100g sand	10 (n=10)	10 (n=10)	8 (n=10)
21mL H_2O/100g sand	9 (n=9)	10 (n=10)	7 (n=7)

The sample sizes for eggs failing to hatch and becoming colonised by fungi were too small to allow for statistical analysis, but data are given in Table 9.3. *F. solani* was isolated from the exterior of both failed control and inverted eggs, but *F. oxysporum* appeared on inverted eggs only. One inverted egg had a mixed culture of these two fusaria (see Table 9.4).

Table 9.3 The number of failed control green turtle eggs colonised by fungi under varying thermal and hydric incubation regimes.

	Temperature		
Moisture	26°C	28°C	30°C
7mL H_2O/100g sand	5 (n=6)	1 (n=2)	2 (n=4)
14mL H_2O/100g sand	4 (n=4)	-	1 (n=1)
21mL H_2O/100g sand	2 (n=2)	1 (n=2)	0 (n=1)

Table 9.4 The proportion of green turtle eggs colonised by *F. oxysporum* and *F. solani* during artificial incubation experiments investigating the influence of thermal and hydric conditions on fungal colonisation.

Species of Fungi	Failed Control Eggs (n=22)	Inverted Eggs (n=86)
None	27%	6%
F. oxysporum	0%	91%
F. solani	73%	2%
F. oxysporum + *F. solani*	0%	1%

9.3.2 The Influence of the Thermal and Hydric Environment on the Linear Growth Rate of Nest Mycobiota

The linear growth rate of *F. oxysporum*, *F. solani* and *P. boydii* at the experimental thermal and hydric regimes are given in Tables 9.5-9.7 and Figures 9.1-9.3. Both temperature and moisture had a significant effect on the linear growth rate of all fungi (see Table 9.8), and there was significant interaction between the 2 factors in all analyses (see Table 9.8), indicating synergistic effects.

Table 9.5 The mean linear growth rate of *F. oxysporum* under various thermal and hydric regimes.

	Linear Growth Rate (mm day^{-1})			
Water Potential (kPa)	26°C	28 °C	30 °C	32°C
-173	6	7	5	6
-468	7	8	6	6
-922	7	7	6	7
-1372	7	8	6	7
-1821	7	7	6	7
-2269	6	7	5	6
-2716	5	5	6	5
-3169	4	5	5	5

Table 9.6 The mean linear growth rate of *F. solani* under various thermal and hydric regimes.

	Linear Growth Rate (mm day^{-1})			
Water Potential (kPa)	26°C	28 °C	30 °C	32°C
-173	5	6	5	5
-468	5	6	6	6
-922	5	6	6	5
-1372	5	6	5	5
-1821	5	6	6	5
-2269	4	6	5	5
-2716	4	5	4	4
-3169	3	5	3	4

Table 9.7 The mean linear growth rate of *P. boydii* under various thermal and hydric regimes.

	Linear Growth Rate (mm day^{-1})			
Water Potential (kPa)	26°C	28 °C	30 °C	32°C
-173	2	2	4	3
-468	3	3	4	4
-922	3	3	5	5
-1372	3	3	4	4
-1821	2	3	3	3
-2269	1	1	2	2
-2716	1	0	1	1
-3169	0	0	0	0

Table 9.8 *F* statistics of Model II two-factor analyses of variance determining the influence of temperature and moisture on the linear growth rate of *F. oxysporum*, *F. solani* and *P. boydii*.

Fungi	Temperature	Water Potential	Interaction
F. oxysporum	$F_{0.05(2),3,21}$=4.986, P<0.02	$F_{0.05(2),7,21}$=7.460, P<0.001	$F_{0.05(2),21,63}$=8.509, P<0.001
F. solani	$F_{0.05(2),3,21}$=31.153, P<0.001	$F_{0.05(2),7,21}$=26.139, P<0.001	$F_{0.05(2),21,63}$=2.187, P<0.001
P. boydii	$F_{0.05(2),3,21}$=14.128, P<0.001	$F_{0.05(2),7,21}$=47.571, P<0.001	$F_{0.05(2),21,63}$=8.611, P<0.001

Figure 9.1 The Influence of Temperature and Moisture on the Mean Linear Growth Rate of *F. oxysporum*.

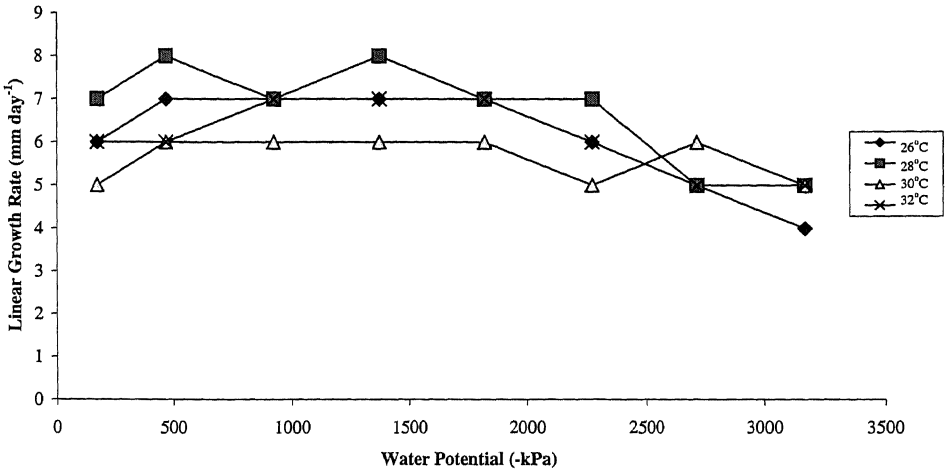


Figure 9.2 The Influence of Temperature and Moisture on the Mean Linear Growth Rate of *F. solani*.

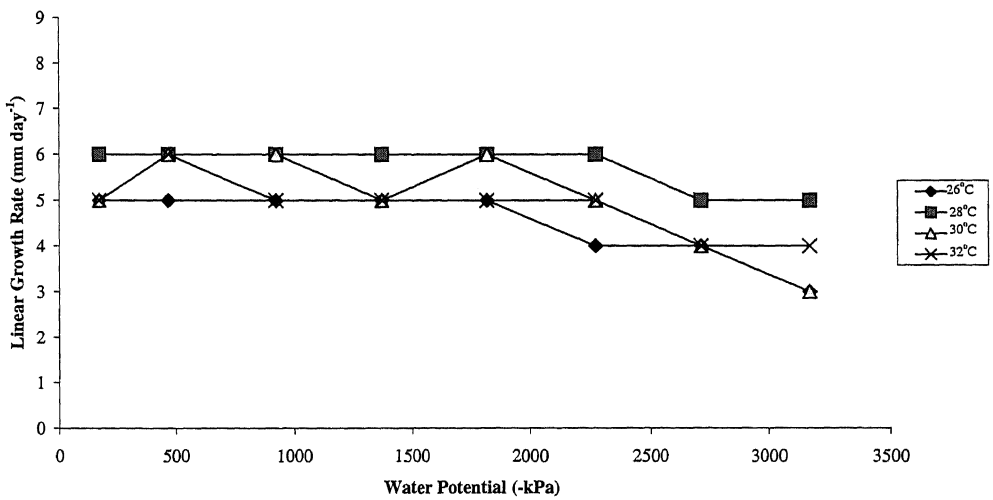
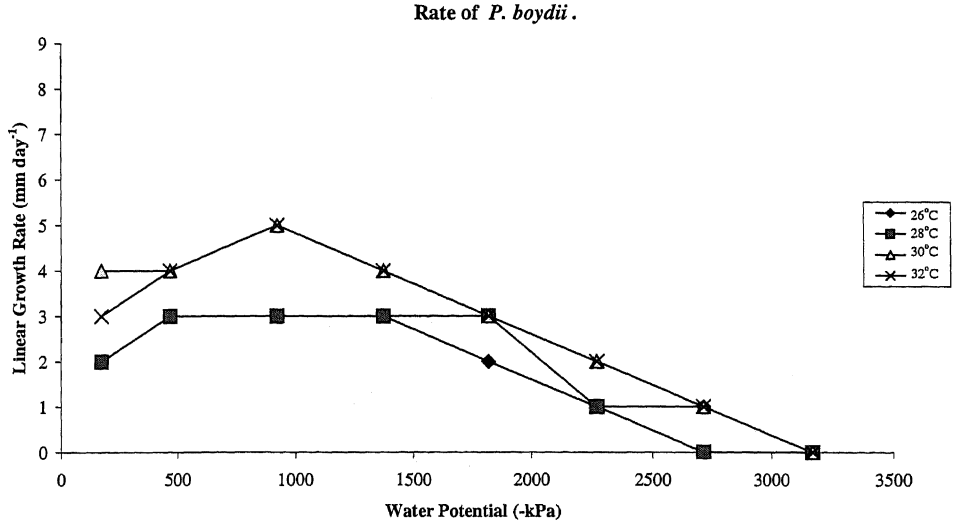


Figure 9.3. The Influence of Temperature and Moisture on the Mean Linear Growth Rate of *P. boydii*.



Within the combinations of temperature and water potential used, the linear growth rates of *F. oxysporum* and *F. solani* were greatest at 28°C between - 173 and - 2268kPa. Optimal growth of *P. boydii* occurred at 30-32°C between - 173 and - 1372kPa. The fastest growth rate under any given conditions was typically *F. oxysporum*, followed by *F. solani* then *P. boydii*. With the exception of *P. boydii*, with no growth at - 2716kPa at 28°C and - 3168.9kPa at 26-32°C, all fungi showed some growth at every thermal and hydric regime tested.

9.4 DISCUSSION

In this experiment, the lowest hatch success occurred at the lowest water content and lowest temperature. Substrate water potential had the greatest influence, which is not surprising since eggs were incubated well within the thermal tolerance range of sea turtles (25-33°C, Miller 1982). Below optimum moisture has also had the greatest influence on hatch success in several other studies (McGehee 1990, Mortimer 1990, Hewavisenthi and Parmenter 2001).

Colonisation of inverted green turtle eggs by *F. solani* was independent of moisture but significantly affected by thermal conditions present during incubation. Since *F. oxysporum* was detected from only 2% of all eggs with fungi and *P. boydii* was not isolated, the same conclusions cannot be drawn for these species.

F. solani grew between 26-32°C and - 173.23 to - 3168.9kPa, although linear growth rate was significantly affected by temperature and available moisture (see Table 9.8). *F. oxysporum* and *P. boydii* were not isolated from failed sea turtle eggs in the incubation experiment, but in the absence of other factors their growth

should be affected, but not completely inhibited, by the thermal and hydric microclimatic conditions of the sea turtle nest, with two exceptions; there was no growth of *P. boydii* at 28°C at - 2716kPa or at any temperature at - 3169kPa. However, as this water potential is believed to be at the upper threshold of tolerance for sea turtle eggs (Hewavisenthi 1999) these conditions are very unlikely to occur often other than at Peak Is..

The linear growth rate of fungi will determine the speed at which hyphae spread from egg to egg during the invasion of sea turtle nests. Once a failed egg within the egg mass has been colonised and serves as a nutrient focus (see Chapter 5) the progress of hyphae to adjacent eggs would be fastest around 28°C and - 173.23kPa to - 1372kPa for *F. oxysporum* and *F. solani* and 30-32°C at the same water potential for *P. boydii.* Water potential does not inhibit the growth of *F. oxysporum* and *F. solani*, but *P. boydii* does not grow at - 2716kPa (28°C only) or - 3168.9kPa (26-32°C).

CHAPTER 10

THE INFLUENCE OF EMBRYONIC DEVELOPMENT ON EGG SUSCEPTIBILITY TO FUNGAL COLONISATION

INTRODUCTION

Fungal colonisation of a sea turtle nest begins at a non-viable egg that acts as a nutrient source or focus for subsequent hyphal spread to adjacent eggs (see Chapter 6). The timing of this initial egg failure is likely to determine the vulnerability of the remainder of the nest to mycobiota in several ways. First, it will determine the period remaining prior to hatching and thus the time available for fungal colonisation of the non-viable egg and subsequent spread through the nest. Second, the stage of embryonic development may also determine the effectiveness of the chemical and physical defence mechanisms of the remaining eggs against infection (reviewed in Chapter 11).

To determine stage-specific periods of vulnerability and to investigate the relative pathogenicity of each known nest isolate, eggs were exposed to *F. oxysporum*, *F. solani* and *P. boydii* at different times during their incubation period.

PROCEDURE

To determine the potential for stage-specific vulnerability to fungal invasion, eggs were exposed to fungi at various stages during incubation. Green turtle eggs (45 eggs randomly taken from each of 10 clutches) were collected at Heron Is., maintained at 8°C for 48hrs at the Heron Island Research Station and then

transported to Central Queensland University. Eggs were incubated in a controlled-temperature room at 28°C to predetermined developmental stages (see Table 10.1). The stages were visually assessed (half white-spot, full white-spot) or calculated from known incubation periods at 29°C (64 days- Miller 1982; therefore the 1st trimester ends at day 21 and the 2nd trimester at day 42).

Table 10.1 Allocation of green turtle clutches to developmental stages during artificial incubation studies used to determine the potential for stage-specific vulnerability to fungal colonisation of sea turtle eggs.

Developmental Stage	Clutch
Day 0 (oviposition) Day 7 (half white-spot) Day 14 (Full white-spot)	Clutch 1-5
Day 21 (end 1st trimester) Day 42 (end 2nd trimester) Day 60 (prior to pipping)	Clutch 6-10

Three eggs per clutch were assigned to each combination of developmental stage and treatment. These were incubated as isolated groups in 25x19x9cm plastic containers on a 2cm substrate of heat-sterilised sand collected from average nesting depth (55cm) at the Heron Is. nesting beach. The egg container was covered with plastic film and a perforated lid to allow exchange of respiratory gases but not fungal spores. Substrate moisture was maintained by sub-surface trickle irrigation with sterile, distilled water. Eggs were spaced so they did not touch adjacent eggs or the container surface, thus preventing the transfer of fungi. Eggs that died prior to the allocated stage of treatment were removed from the incubation container so as not to confound results.

At their allocated developmental stage, eggs were inverted and agitated in an attempt to cause embryo mortality (and allow subsequent fungal colonisation of the egg) and then either exposed to one of 4 treatments (a spray with sterile distilled water, *F. oxysporum* spore suspension, *F. solani* spore suspension or *P. boydii* spore suspension) or left untouched as a control. Spore suspensions were prepared by harvesting spores from axenic cultures of each fungus that had previously been incubated on PDA for 7 days at 28°C, and the concentration adjusted to 10^3 spores per mL. Laboratory experience had indicated *P. boydii* was the least and *F. solani* the most invasive, so the treatment sequence least likely to result in cross-contamination was followed: sterile distilled water, *P. boydii* spore suspension, *F. oxysporum* spore suspension then *F. solani* spore suspension. A 1mL sample of each spore solution was plated with 15mL of PDA and incubated at 28°C to monitor colony formation and ensure spore suspension viability.

After treatment, eggs were left to incubate until hatching and the hatchlings released into Keppel Bay after absorption of their residual yolk sac. Swabs of fungus from the exterior of unhatched eggs were cultured and identified following the procedures described in Chapter 4.

RESULTS

Despite the heat-sterilisation of the substrate, and application of different treatments (control, sterile distilled water, *F. oxysporum* spore suspension, *F. solani* spore suspension or *P. boydii* spore suspension), all of the failed eggs in this study were colonised by *F. solani*. However, all of the spore suspensions produced the

parent colony when incubated on PDA and there was no growth from the sterile distilled water.

The sample size of failed eggs within each treatment was dependent upon the survival of inverted eggs at each developmental stage (see Table 10.2). Hatch success after disturbance of green turtle eggs at various developmental stages (see Table 10.2) was similar to that calculated for green turtle eggs by Parmenter (1980) and loggerhead turtle eggs by Limpus (1979). Therefore, sample size was reduced as the embryo became more tolerant of disturbance.

Table 10.2 The influence of developmental stage on the hatch success of disturbed eggs and colonisation of failed eggs by *F. solani*.

Developmental Stage (sample size)	Hatch Success of Disturbed Eggs (%)	Fungal Colonisation of Failed Eggs (%)
Oviposition (n=30)	90	100
Half white-spot (n=65)	13	100
Full white-spot (n=74)	0	100
End of 1st trimester (n=66)	29	100
End of 2nd trimester (n=64)	89	100
End of 3rd trimester (n=67)	91	0

All eggs in all treatments at each developmental stage were colonised by fungi, with the exception of eggs inverted and sprayed immediately prior to pipping. At this stage, there were no visible signs of fungi on the egg exterior before hatching occurred.

DISCUSSION

All eggs were colonised by fungi after embryo mortality at any developmental stage investigated prior to pipping. Presumably, when fungal spores

come into contact with eggs that had failed immediately prior to pipping, there was insufficient time to colonise these eggs and spread to adjacent viable eggs prior to the emergence of the hatchlings several days later. The experimental design (based on the number eggs allowed to be taken under permit) did not allow egg vulnerability between the end of the 2nd trimester and pipping to be more closely measured. However, fungal colonisation late in the 3rd trimester is likely to be a lesser threat than earlier during incubation as the embryos are closer to completing their development and less time is available for fungi to spread from a host egg to adjacent eggs. The synchronous emergence pattern of sea turtle nests also prevents a staggered hatching of eggs or asynchronous emergence, which would render viable but unhatched eggs still vulnerable to fungal colonisation.

F. solani was able to colonise sea turtle eggs that failed at any embryonic stage prior to that immediately before pipping. Although eggs were also exposed to *F. oxysporum* and *P. boydii*, the same conclusion cannot be drawn for these fungi as they were not isolated from any failed eggs. Since all spore suspensions proved viable when plated with PDA, it is presumed the substrate was not completely heat-sterilised and colonisation of the turtle eggs probably occurred from spores or hyphae remaining in the substrate and not from the artificially applied spore suspension.

Therefore, green turtle eggs appear vulnerable to colonisation by *F. solani* after embryo mortality at any time prior to pipping. The proportion of the clutch subsequently infected as the fungi spreads from the non-viable host egg to adjacent

eggs will be dependent upon the time remaining before hatching, as failed eggs appear to have no residual chemical or physical defences against fungal invasion.

CHAPTER 11

POTENTIAL CHEMICAL AND PHYSICAL DEFENCES AGAINST FUNGAL INVASION OF SEA TURTLE EGGS

In the absence of a developed immune response or maternal antibodies, the developing egg must rely on non-specific defences against microbial invasion. Board and Fuller (1974) suggested avian cleidoic eggs are shielded from microbial colonisation by a combination of physical barriers, such as the eggshell and shell membranes, together with chemical defences such as the albumen. These same systems are likely to play a defensive role during the incubation of sea turtle eggs. In addition, at oviposition chelonian eggs are covered with a mucus that is believed to have anti-pathogenic properties (C.J. Limpus, pers.comm.).

There may be inter-specific differences in the effectiveness of the mucus, albumen and eggshell in protecting sea turtle eggs against fungal colonisation. This would result in inter-specific variation in fungal–related egg mortality, as observed between the green and loggerhead turtles nesting on Heron Is. and Wreck Is. (see Chapter 3).

11.1 MUCUS SECRETED DURING OVIPOSITION

11.1.1 INTRODUCTION

A clear mucus which coats the eggs is secreted from the cloaca during oviposition in green (Bustard and Greenham 1969), hawksbill (Carr *et al.* 1966), loggerhead and flatback (Bustard *et al.* 1975) sea turtles (see Figure 11.1.1).

Figure 11.1.1 Sea Turtle Mucus Secreted During Oviposition (photo of flatback turtle courtesy of C.J. Parmenter/L.J. Parmenter).

This glyco-protein secretion is produced by the surface epithelium of the shell-forming section of the oviduct (Aitken and Solomon 1976b). Freshwater turtle eggs washed free of their mucus succumb to infection more easily than coated eggs (Ewert 1985), suggesting it may have anti-pathogenic properties.

11.1.2 PROCEDURE

Mucus was collected from 10 individuals of each of the following species; loggerhead turtles nesting at Mon Repos; green turtles nesting at Heron Is.; hawksbill turtles nesting at Milman Is.; and, flatback turtles nesting at Peak Is.. The rear of the nest was widened by hand as the turtle completed egg-chambering to allow a sterile 20mL specimen jar to be inserted and held beneath the ovipositor so mucus could be collected as each individual turtle commenced oviposition. Samples were stored at -5°C prior to use.

To determine possible anti-pathogenic properties of turtle mucus, the fungi *F. oxysporum*, *F. solani* and *P. boydii*, all of which are known to colonise sea turtle eggs (see Chapter 3), were exposed to mucus from each species of sea turtle. Initial spore suspensions were made by harvesting spores from established monoculture plates of each fungus that had been incubated on PDA at 28°C for 7 days. A solution of 10mL of sterile distilled water and 0.1% Tween 20 was poured onto each plate and the spores dislodged by gentle disruption of the colony surface with a sterile "hockey stick". The spore suspension was poured into a sterile McCartney bottle, its spore concentration adjusted to 10^3 spores mL^{-1} with sterile distilled water, and then poured onto sterile PDA plates so it covered the entire surface, after which the excess

was poured off. This formed axenic lawn plates of *F. oxysporum*, *F. solani* and *P. boydii* spores.

A well was punched into the centre of each plate with a heat-sterilised 10mm cork-borer. Approximately 1mL of mucus that had been thawed to room temperature was added to each well. Mucus samples from the 10 individuals of each turtle species were tested in triplicate (i.e. 3 plates each) for inhibition of *F. oxysporum*, *F. solani* and *P. boydii* spore germination. Plates were incubated at ambient laboratory temperature for 7 days for observation and comparison with spore germination patterns from equal numbers of replicates of lawn plates without wells, lawn plates with empty wells, and lawn plates with wells containing sterile distilled water that had been incubated simultaneously under the same conditions.

11.1.3 RESULTS

Mucus from all replicates of the 10 individuals of each of the 4 turtle species affected spore germination of all species of fungi. Spores of *F. oxysporum*, *F. solani* and *P. boydii* were inhibited from germinating for 3 days in a zone of up to 5mm around each well containing mucus (see Figure 11.1.1) while spores on the remainder of the plate germinated. During this time the mucus level in the well fell as it diffused into the agar. Limited germination commenced in the previously inhibited zone after Day 3. Lawn plates without wells, or with wells not containing mucus did not develop a zone of inhibition at any time (see Table 11.1.1).

Figure 11.1.2 Diagrammatic Representation of Fungus Spore Germination Inhibited By Sea Turtle Mucus (figure not to scale).

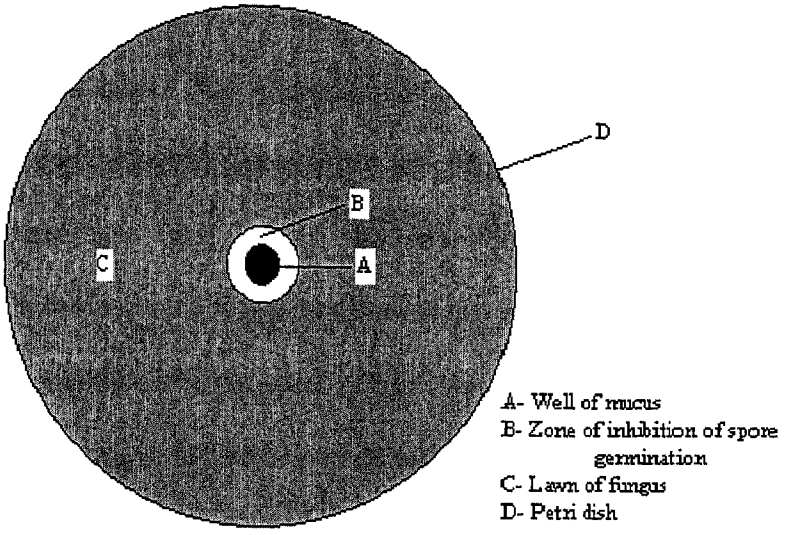


Table 11.1.1 Germination (+ or -) of *F. oxysporum* (Fo), *F. solani* (Fs) and *P. boydii* (Pb) spores in the presence of sea turtle mucus.

	Treatment			
Turtle	Control	Empty Well	Well with Water	Well with Mucus
Loggerhead	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Green	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Hawksbill	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Flatback	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)

11.1.4 DISCUSSION

These results show that spore germination of *F. oxysporum*, *F. solani* and *P. boydii* is inhibited by mucus secreted during the oviposition of loggerhead, green, hawksbill and flatback turtles eggs. The mechanism of inhibition has not been determined, but it is likely due to the action of enzymes and other proteins, as occurs in other oviparous vertebrates (see Table 11.1.2). Inhibition of spore germination decreased after 3 days, probably due to simple dilution of the active agents as the mucus in the well slowly leached into the surrounding agar. Alternatively, the mucus proteins may only have a short active life and have reduced effectiveness after 3 days in these conditions.

In a natural situation, the mucus that covers the turtle egg at oviposition may prevent *F. solani*, *F. oxysporum* and *P. boydii* spores from germinating and potentially colonising viable eggs. Mucus dries on the egg within several days (pers.ob.). and it is not known whether the anti-pathogenic properties continue after this time. There was no detectable difference between loggerhead and green turtle mucus in ability to inhibit spore germination so it is unlikely that differences in mucus antibiotic actions are contributing to the inter-specific variation in egg mortality observed at Heron Is. and Wreck Is..

Table 11.1.2 Proteins with anti-pathogenic properties that are active in avian albumen and the fertilisation envelope of fish.

Protein	Function
Avidin[1]	combines with biorin so it is unavailable for microbial utilisation
Avoprotein[1]	combines with riboflavin so it is unavailable for microbial utilisation
Cellulase[2]	degrades cellulose in fungus cell walls
Chitinase[2]	degrades chitin in fungus cell walls
Dextranase[2]	degrades dextran in fungus cell walls
Laminarase[2]	degrades laminin in fungus cell walls
Lichenase[2]	degrades lichenin in fungus cell walls
Mannase[2]	degrades mannan in fungus cell walls
Ovoinhibitor[1]	inhibits fungal proteases
Ovomucoid[1]	inhibits trypsin
Ovotransferrin[1]	chelates Fe^{3+}, Cu^{2+} and Zn^{2+} to result in pH 9.6 in avian albumen to cause alkaline shock to pathogens
Protease[2]	degrades proteins in fungus cell walls
Xylanase[2]	degrades xylan in fungus cell walls

[1]Board and Fuller 1974
[2]Kudo and Teshima 1991

11.2 SEA TURTLE EGG ALBUMEN

11.2.1 INTRODUCTION

Sea turtle albumen is produced in the magnum, or albumen producing segment, of the oviduct. Bacteriocidal and fungicidal properties of steppe tortoise, *Testudo horsfieldi*, albumen have been reported by Movchan (1964, 1966, 1967), including temporal variation in activity, but there have been no equivalent studies on sea turtle albumen. If sea turtle albumen has anti-fungal properties, inter-specific variation in effectiveness between green and loggerhead turtles may contribute to the differential hatch success observed between these species at Heron Is. and Wreck Is..

11.2.2 PROCEDURE

To determine the anti-fungal properties of sea turtle albumen, 1 egg was taken from each of the flatback, green, hawksbill and loggerhead turtles from which mucus was collected in Section 11.1. Eggs were collected by gloved hand directly from the

ovipositor and placed directly in sterile plastic bags that were labelled and stored at - 5°C prior to analysis.

To determine potential temporal variation in the anti-fungal properties of sea turtle albumen, an entire clutch of green turtle eggs was collected directly from the ovipositor and temporarily stored in sterile plastic bags. At the completion of laying, the eggs were carried to the Heron Island Queensland Parks and Wildlife Station where they were incubated in a styrofoam box on a 2.5cm of heat sterilised sand. Each day, a single egg was transferred from the box directly to a freezer for euthanasia at –5°C. Sampling proceeded until the remainder of the clutch hatched. Sampled eggs were maintained frozen until they were transported to Central Queensland University for analysis.

Eggs were thawed to room temperature in a BioHazard cabinet, opened and the albumen separated from the other egg components then stored in sterile 20mL specimen jars. Lawn plates of *F. oxysporum*, *F. solani* and *P. boydii* with wells containing albumen and appropriate controls were prepared and incubated as in Section 11.1.

11.2.3 RESULTS

F. oxysporum, *F. solani* and *P. boydii* spore germination was inhibited by sea turtle albumen from eggs at oviposition in the same way as occurred by mucus from the same turtles. Albumen from all replicates of the 10 individuals of each of the 4 turtle species affected spore germination of all species of fungi. Spores were inhibited from germinating for 3 days in a zone of up to 5mm around each well

containing albumen while spores on the remainder of the plate germinated. As previously observed for mucus, albumen was lost from the wells as it diffused into the agar and limited germination commenced in the previously inhibited zone after Day 3. Plates incubated without wells or without albumen in the wells did not develop a zone of inhibition at any time (see Table 11.2.1).

Table 11.2.1 Germination (+ or -) of *F. oxysporum* (Fo), *F. solani* (Fs) and *P. boydii* (Pb) spores in the presence of sea turtle albumen.

	Treatment			
Turtle	Control	Empty Well	Well with Water	Well with Albumen
Loggerhead	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Green	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Hawksbill	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)
Flatback	Fo+ (n=10)	Fo+ (n=10)	Fo+ (n=10)	Fo- (n=10)
	Fs+ (n=10)	Fs+ (n=10)	Fs+ (n=10)	Fs- (n=10)
	Pb+ (n=10)	Pb+ (n=10)	Pb+ (n=10)	Pb- (n=10)

Tests to determine temporal variation in the anti-fungal properties of green turtle albumen during incubation were hampered by bacterial contamination of lawn plates but albumen sampled at Days 1-9 and Days 30-46 inhibited spore germination by *F. oxysporum*, *F. solani* and *P. boydii* (see Figure 11.2.1). The magnitude of the zone of inhibition of spore germination varied with day of incubation (see Figure 11.2.1). Tests of albumen collected during the remainder of the incubation period had to be discarded. Since only 1 clutch of eggs was collected, the experiment could not be repeated.

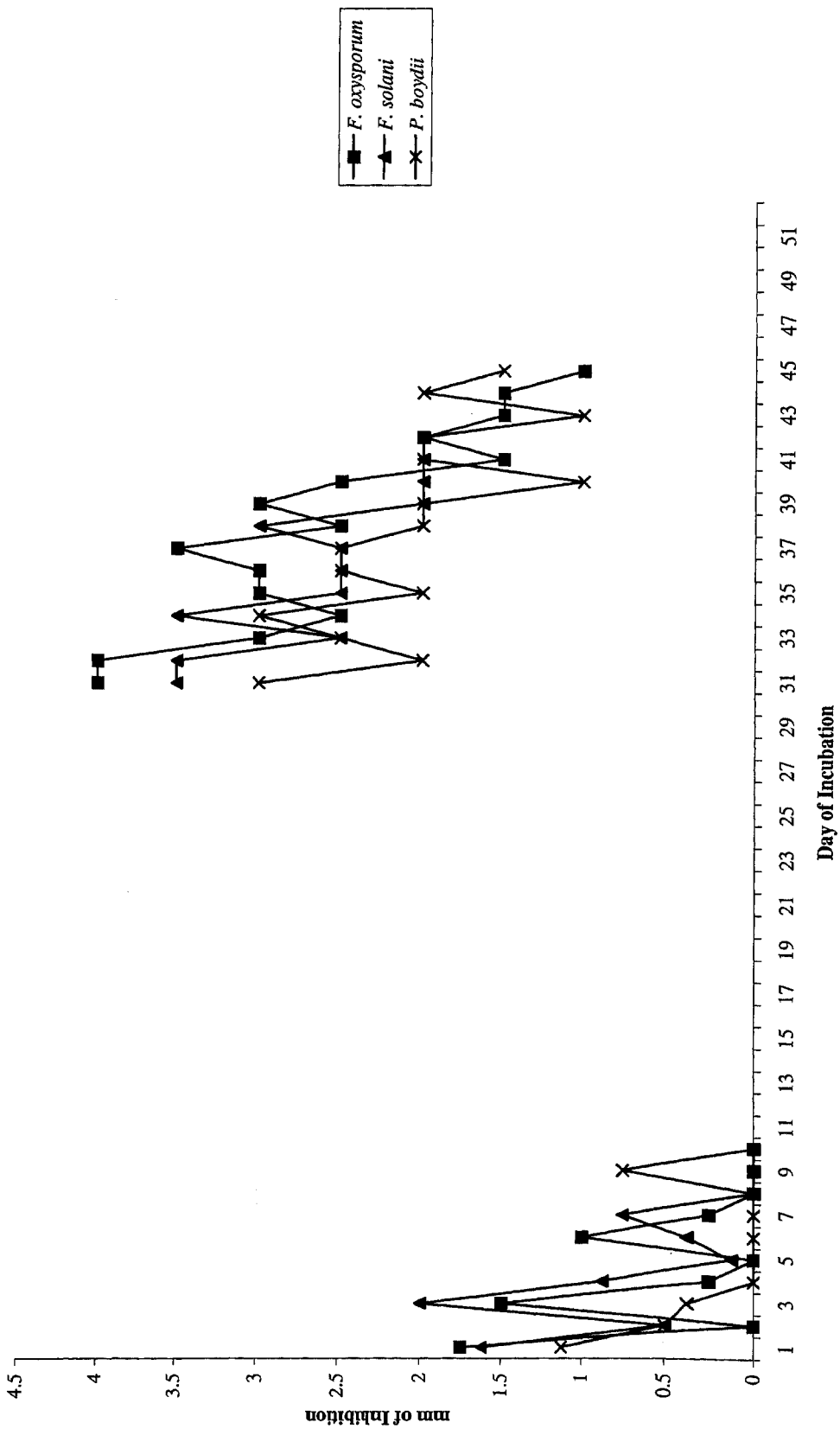


Figure 11.2.1 Temporal Variation in Inhibition of Fungus Spore Germination by Green Turtle Albumen.

11.2.4 DISCUSSION

At oviposition, flatback, green, hawksbill and loggerhead turtle albumen had anti-fungal properties that inhibited germination of *F. oxysporum*, *F. solani* and *P. boydii* spores. There was no difference detected in the effectiveness of green and loggerhead turtle albumen at oviposition in inhibiting spore germination, therefore, it is unlikely that differences in albumen antibiotic actions at oviposition contributes to the inter-specific variation in egg mortality observed at Heron Is. and Wreck Is..

Green turtle albumen inhibited spore germination during Days 0-9 and 30-46 of incubation. Its effectiveness during the remainder of the incubation period is unknown as bacterial contamination destroyed test plates. However, the magnitude of its effect at Day 30 suggests the anti-fungal properties are likely to continue between Days 10-29.

The temporal variation in inhibition of fungus spores during incubation suggests anti-fungal properties of green turtle mucus are related to embryo development. However, without data for the missing periods of incubation, the role of the embryo in the chemical defences of the egg is speculatory.

As both green and loggerhead turtle albumen at oviposition inhibited spore germination, the anti-fungal properties of loggerhead albumen may continue throughout incubation, as demonstrated for green turtle albumen. Permit restrictions prevented the collection of a clutch of loggerhead turtle eggs to determine temporal variation in their effectiveness, so a comparison of the 2 species is not possible. However, it would seem unlikely that the albumen of only one species would possess

anti-fungal properties during incubation, so it is not anticipated that variation in sea turtle albumen enzymes is contributing to inter-specific variation in fungal colonisation of eggs at Heron Is. and Wreck Is..

The precise anti-fungal action of sea turtle albumen was not investigated. Since egg albumen has a water potential of –900kPa (Ackerman 1991) and *Fusarium* spp. spores are not inhibited from germinating until <-1200kPa, osmotic stress is unlikely to play a role. The albumen probably contains proteins similar to those in Table 11.1.2.

11.3 THE ULTRA-STRUCTURE OF SEA TURTLE EGGSHELL

11.3.1 INTRODUCTION

The term "eggshell" refers to all layers of the freshly oviposited egg external to the albumen. The flexible shelled eggs of sea turtles consist of a well-defined inorganic, calcareous layer approximately the same thickness as the adjacent inner, organic shell membrane (Ewert 1979, Packard 1980, Solomon and Baird 1979). Its function is to separate the developing embryo from the external environment while still allowing the transfer of moisture and respiratory gases required during embryogenesis.

Eggshell formation occurs in the oviduct. The caudal shell forming segment of the oviduct appears to secrete both the protein-carbohydrate shell membrane and the calcium of the shell matrix (Solomon and Baird 1976, 1979). Eggs of the green (Miller 1982) and olive ridley (Owens 1980) sea turtles are contained in a slightly calcified shell membrane within 3 days of ovulation. Shell thickness then increases

rapidly until 8-9 days post-oviposition when calcification is complete and the egg is structurally ready for oviposition (Miller 1982).

The calcareous component of reptilian eggshells is calcium carbonate, present in the aragonite form in chelonian eggs (Young 1950, Baird and Solomon 1979, Packard and Packard 1979, Packard 1980). Calcite may be present in eggs produced by farmed green sea turtles (Baird and Solomon 1979, Solomon and Baird 1980), possibly due to dietary imbalances (Solomon and Baird 1980).

There is general consensus as to the structural similarity of eggshell from wild sea turtle species (Solomon and Baird 1976, Solomon and Baird 1977, Baird and Solomon 1979, Packard *et al.* 1982, Hirsch 1983, Acuña-Mesén 1984, Solomon and Watt 1985, Schleich and Kästle 1988, Acuña-Mesén 1989, Chan and Solomon 1989, Sahoo *et al.* 1996a, 1996b - see Table 11.3.1). The aragonite crystals, in the form of micronodules, spicules and blocks, are loosely organised to form an open matrix. The organic layer consists of inter-woven, branched fibrils that have a netted appearance.

However, Carthy (1992) described pore-like structures on the inner surface of loggerhead turtle shell membrane. These were not observed by Packard *et al.* (1982) or Schleich and Kästle (1988). As higher eggshell porosity may allow for easier or greater fungal penetration of the sea turtle egg, this could explain the inter-specific variation in fungal colonisation of eggs and hatch success that occurs between green and loggerhead sea turtles at Heron and Wreck Is.. Consequently, eggshell from

loggerhead turtles nesting in eastern Australia was compared with that of other turtle species to determine if it had increased porosity.

Table 11.3.1 Descriptions of sea turtle eggshell ultra-structure.

Turtle	Source	Type of Egg Examined
Loggerhead	Packard *et al.* 1982	Unknown
	Schleich and Kästle 1988	Hatched and failed
	Carthy 1992	Hatched
Green	Solomon and Baird 1976	Oviductal and oviposited
	Solomon and Baird 1977	Oviductal and oviposited
	Baird and Solomon 1979	Oviposited and hatched
Leatherback	Solomon and Watt 1985	Oviposited
	Chan and Solomon 1989	Hatched and failed
Hawksbill	Acuña-Mesén 1989	Oviposited eggs
Kemp's Ridley	Packard *et al.* 1982	Unknown
	Hirsch 1983	Unknown
Olive Ridley	Acuña-Mesén 1984	Unknown
	Sahoo *et al.* 1996a	Oviposited eggs
	Sahoo *et al.* 1996b	Oviposited eggs

11.3.2 PROCEDURE

To compare the ultrastructure of eggs among sea turtles nesting in eastern Australia, eggs were collected directly from the ovipositor of nesting flatback turtles at Peak Island, loggerhead turtles at Mon Repos, hawksbill turtles at Milman Island and green turtles at Heron Island, and immediately frozen to –5°C for storage. One egg from each of 5 turtles of each species was examined by a Jeol JSM-5300LV Scanning Electron Microscope. Prior to analysis, eggs were thawed to room temperature and their contents removed. The eggshell was air-dried and fragments mounted on double-sided carbon tape. Preliminary examination of specimens indicated gold sputter-coating was not required to view the eggshell structure, so uncoated eggshell of each species was examined at an accelerating voltage of 20kV. Transverse sections and both interior and exterior surfaces were photographed with

TMAX 100 ASA black and white film. An external surface view for loggerhead eggshell was unsuccessful.

11.3.3 RESULTS

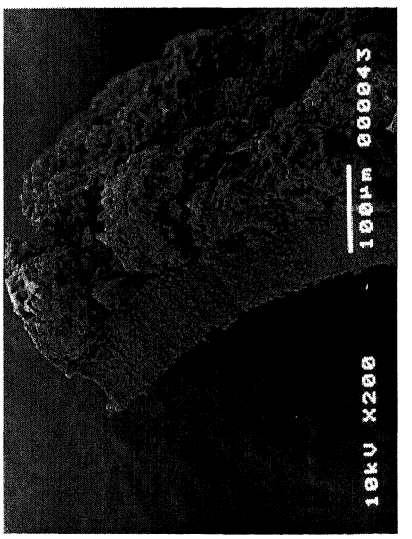
Scanning electron microscope micrographs of flatback, green, hawksbill and loggerhead sea turtle eggshell are presented in Figures 11.3.1-11.3.3. This is the first description of flatback sea turtle eggshell. The arrangement of aragonite crystals in the inorganic layer was similar to that of other sea turtle species, as was the fibril arrangement in the organic layer. Eggshell of all species was of similar width, although difficulties in mounting fragments did not allow accurate measurements to be taken. The organic layer was wider than the inorganic in the transverse section. No pores were detected in the eggshells of any species. The organisation of the inorganic layer resulted in open spaces of varying sizes between adjacent aragonite crystals, however, spaces between fibrils of the organic layer never exceeded 1μm in any species.

11.3.4 DISCUSSION

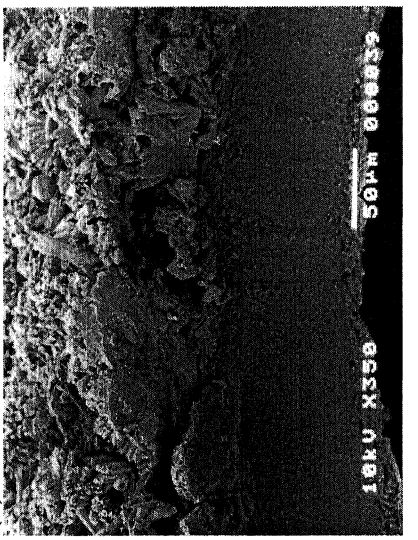
The similarity between eggshell from the 4 species of sea turtle commonly nesting in eastern Australia does not suggest that ultra-structural differences contribute to the greater vulnerability of loggerhead turtle eggs to fungal colonisation observed at Heron Is. and Wreck Is.. Open spaces between crystals in the inorganic layer were obvious, but did not extend through the organic layer as would true pores (see Figure 11.3.1). The arrangement and size of spaces within the inorganic and organic matrices would not appear to allow the uptake of fungal spores or easy penetration by hyphae.

Figure 11.3.1 Scanning Electron Micrographs of Sea Turtle Eggshell- Transverse Section

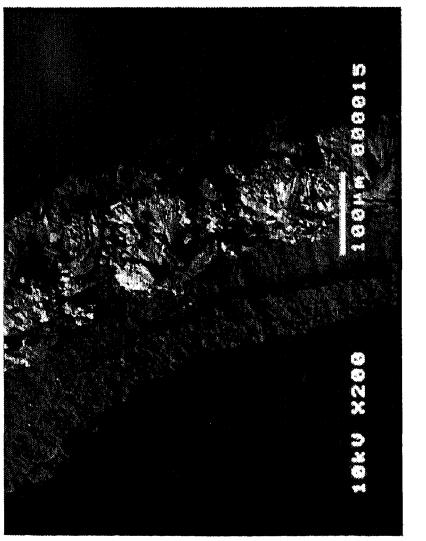
(a) Flatback



(b) Green



(c) Hawksbill



(d) Loggerhead

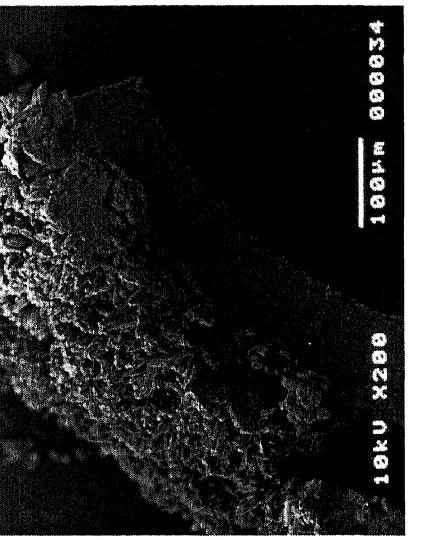
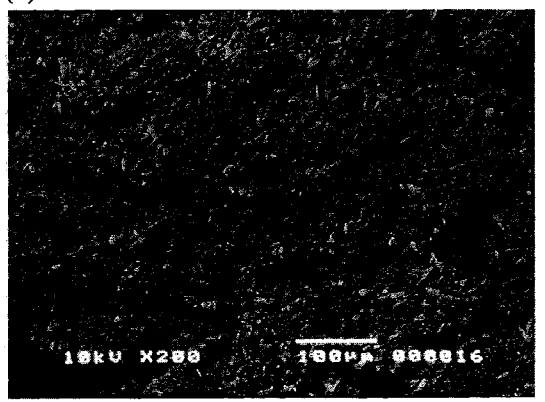
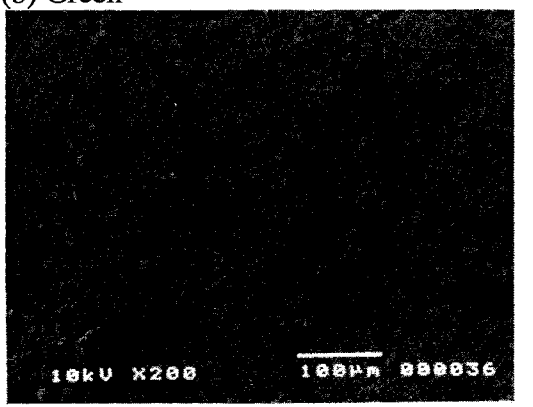


Figure 11.3.2 Scanning Electron Micrographs of Sea Turtle Eggshell- Exterior Surface

(a) Flatback



(b) Green



(c) Hawksbill

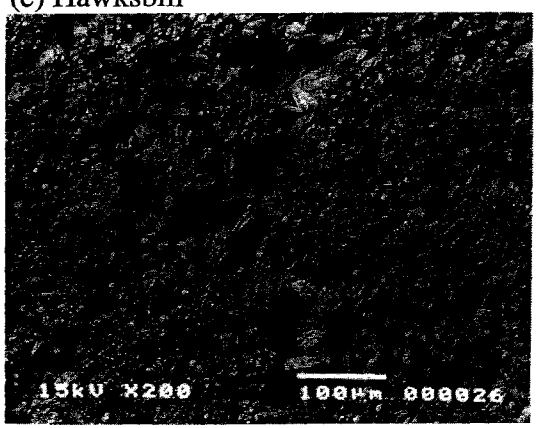
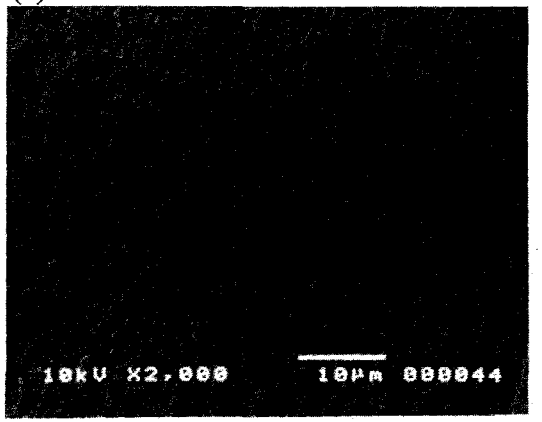
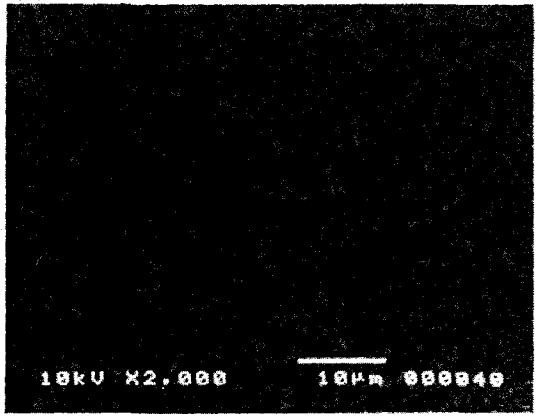


Figure 11.3.3 Scanning Electron Micrographs of Sea Turtle Eggshell- Interior Surface

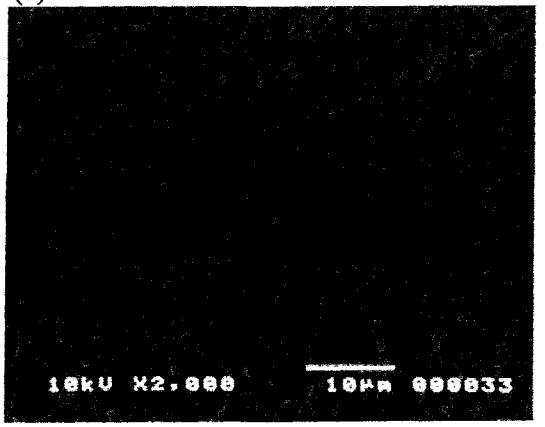
(a) Flatback



(b) Green



(c) Hawksbill



(d) Loggerhead

Table 11.3.2 Dimensions of *F. oxysporum*, *F. solani* and *P. boydii* hyphae and spores.

Fungus	Hyphal Diameter (µm)	Dimensions of Smallest Spores (µm)	Source
F. oxysporum	-	2.2-3.5x5-12	Booth 1971
F. solani	2.5-3	2-4x8-16	Booth 1971
P. boydii	1-3	4-9x6-10	Rippon 1982

CHAPTER 12

THE INFLUENCE OF FUNGAL COLONISATION OF THE SEA TURTLE NEST ON HATCHLING WEIGHT, SIZE AND SCUTELLATION

12.1 INTRODUCTION

The eggshells of hatched sea turtle eggs do not show signs of fungal colonisation, but this does not exclude the possibility that the emerged hatchlings may have been adversely influenced by mycotoxins produced by fungi colonising adjacent, dead eggs within the nest. Fungal metabolites may act as teratogens that have a sub-lethal but physically or physiologically deleterious effect on the hatchlings.

Hatchling fitness is often estimated from hatchling size and/or weight. However, since scute abnormalities may reflect genetic and/or teratogenic factors (Zangerl 1969), patterns of scalation on the shell (carapace and plastron) and head are also used. In the absence of other lethal abnormalities scute abnormalities are unlikely to cause mortality (Miller 1982) but a lower frequency of anomalous scales occurs in hatchlings compared to unhatched embryos (pers.ob) and an even lower frequency occurs in adults (C.J. Parmenter pers.comm., C.J. Limpus pers.comm.). The presence of anomalous scales may, therefore, indicate physical or physiological defects that affect fitness.

Comparing hatchling morphology among nests with varying degrees of fungal colonisation allows the potential influence of mycotoxins on hatchling fitness to be indirectly assessed. The change in the most common species of nest fungi between the 1996/97 and 1997/98 nesting season at Heron Is. (see Chapter 4) also allows a

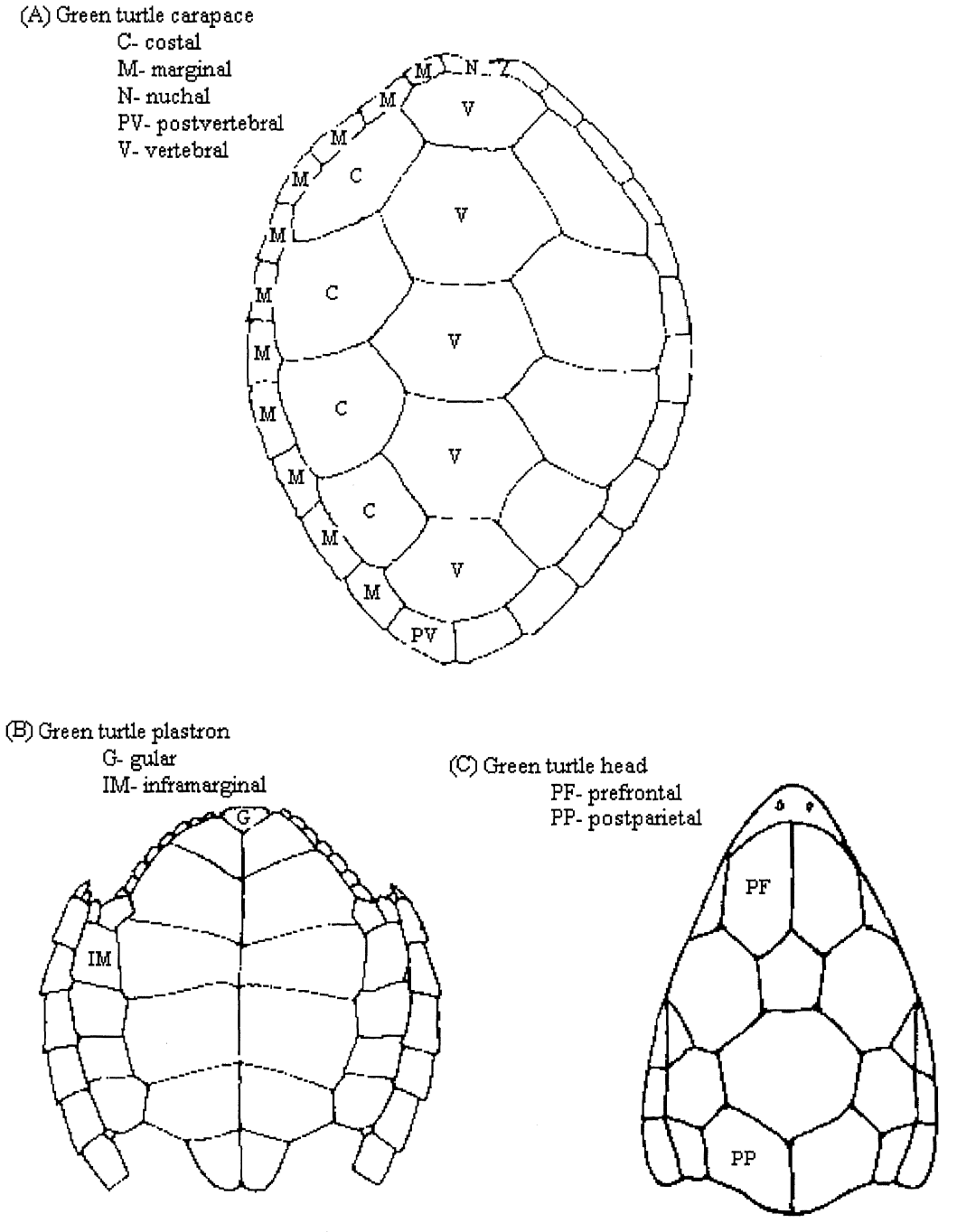
comparison that might suggest whether *F. solani* and *P. boydii* have different effects on the development of turtle embryos.

12.2 PROCEDURE

To determine the occurrence of scale anomalies in sea turtle neonates, 8-10 green turtle hatchlings were selected at random from each clutch during emergence or while they were crossing the beach at Heron Island. In the 1996/97 nesting season 287 hatchlings from 29 clutches were immediately weighed (to 0.01g) on an electronic balance and their straight carapace lengths (SCL) measured with vernier calipers (to 0.1cm). The number of nuchal, vertebral, postvertebral, costal, marginal, gular and inframarginal scales of the shell and postocular, preocular, prefrontal and postparietal scales of the head (see Figure 12.1) were counted and recorded. Hatchlings were then released at their point of capture. In the 1997/98 nesting season 312 hatchlings from 32 clutches were also processed in the same way.

Independent sample t-tests were used to compare hatchling SCL and weight between the 2 nesting seasons. Each season's scale count data were analysed separately because the prevalent fungi changed between seasons (see Chapter 4). The number of scales was compared with the normal scale pattern of green turtles (Marquez 1990) and the number of sub-and super-numerary scales determined for each scale type. Each hatchling was then assigned to one of the following categories: 0, 1, 2, 3, 4, 5, 6, 7 anomalous scale types. For example, a hatchling with no anomalous scales was assigned to the category "0", while a hatchling with an extra nuchal and one less vertebral was assigned to the category of "2".

Figure 12.1 Sea Turtle Scalation.



A 2x2 Chi-square analysis was used to compare the proportion of hatchlings in these categories between the 2 seasons. Categories containing zero counts in both seasons were omitted from the analysis since they are unsuitable for inclusion (Zar 1999). A Chi-square with Yates correction for continuity was used to compare the proportion of hatchlings with and without scale abnormalities between the 2 seasons. Spearman's rank correlation was used to determine whether the number of anomalous scale types varied with hatchling SCL, hatchling weight, nest hatch success, the percentage of failed eggs with fungi and percentage of the clutch with fungi.

12.3 RESULTS

Hatchling SCL (t=-6.521, P<0.001, df=597) and weight (t=-6.371, P<0.001, df=597) were significantly lower in the 1996/97 nesting season than in 1997/98 (see Table 12.1). The proportion of scale categories containing anomalous counts (see Table 12.2) also differed significantly between the two nesting seasons (χ^2=44.17, P<0.0001, df=6). A more detailed account of the occurrence of anomalies is given in Tables 12.3 and 12.4. The percentage of hatchlings with scale anomalies was 75% in 1996/97 but decreased significantly (χ^2=42.04, P<0.0001, df=1) to 49% in 1997/98. This was due to the greater occurrence of sub-numerary postoculars (-1 both left and right) and asymmetry of the postparietal in the first season.

Table 12.1 Green turtle hatchling SCL and weight in the 1996/97 and 1997/98 nesting season at Heron Is. (Mean±SD).

	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

Table 12.2 The number of green turtle hatchlings with anomalous scale counts at Heron Is. in the 1996/97 and 1997/98 nesting seasons.

	# Hatchlings	
# of Anomalous Scale Categories	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

Table 12.3 Scale categories of green turtle hatchlings showing anomalous counts in the 1996/97 nesting season at Heron Is..

			% of Hatchlings with Sub-and Super-numerary Scales					
Scale Category		Normal Count	-2	-1	0	+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)*		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)

*0=scale/s symmetrical; +1=scale/s asymmetrical

Note: rounding may result in category totals not equalling 100%

Table 12.4 Scale categories of green turtle hatchlings showing anomalous counts in the 1997/98 nesting season at Heron Is..

			% of Hatchlings with Sub-and Super-numerary Scales					
Scale Category		Normal Count	-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*	S	-	-	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)

*0=scale/s symmetrical; +1=scale/s asymmetrical

Note: rounding may result in category totals not equalling 100%

There were no significant correlations in the 1996/97 season. The only significant correlation that occurred in the 1997/98 nesting season was between the number of anomalous scale types and the percentage of failed eggs with fungi (see Table 12.5).

Table 12.5 Spearman rank correlation-coefficients between the number of anomalous scale categories and hatchling/nest characteristics in the 1996/97 and 1997/98 nesting seasons at Heron Is..

	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

12.4 DISCUSSION

Hatchling SCL and weight differed significantly between the 2 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 and ambient temperature (see Chapter 4). Such differences in thermal and hydric micro-climatic conditions are one possible cause of the occurrence of scale anomalies, which decreased significantly from 1996/97 to 1997/98. Although the causative mechanism is unknown, experimental studies have shown that shell abnormalities are often related to temperature and/or moisture levels during incubation (Lynn and Ullrich 1950, Packard *et al.* 1987).

There are two possible reasons for the single significant Spearman rank correlation in Table 12.5. 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 colonised by *P. boydii.* Secondly, since the analyses in Table 12.5 are of 10 separate and independent Spearman rank correlations, under an α=0.05 there is a 63% probability of a Type I error (Zar 1999). Therefore, the significant correlation in the 1997/98 season may 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* colonisation of sea turtle eggs on the development of turtle embryos, and although it is tentatively concluded that any effect of either fungus on abnormal scale counts is weak or non-existent, further work is required to reach a more certain conclusion.

In summary, the number of anomalous scales possessed by a hatchling does not appear to be influenced by nest hatch success and evidence for an effect of fungi was very weak. This may be because mycotoxins produced by *F. oxysporum, F. solani* or *P. boydii* do not diffuse far enough from colonised eggs to affect adjacent untouched eggs. Alternatively, mycotoxins may not have teratogenic effects on developing sea turtle embryos. 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, they should have a similar fitness to those from nests without fungi.

CHAPTER 13

GENERAL DISCUSSION

The fungi *F. oxysporum*, *F. solani* and *P. boydii* have been isolated from failed green, loggerhead, hawksbill and flatback turtle eggs at the Heron Is., Wreck Is., Mon Repos, Milman Is. and Peak Is. rookeries in eastern Australia. Previously, the role of fungi in sea turtle nests had not been established as that of opportunistic contaminants of failed eggs or active infectants of viable eggs. The artificial incubation of green turtle eggs in this study allowed patterns of egg mortality and fungal colonisation to be observed. Fungus first appeared on an egg that had failed from other natural causes and, presumably, acted as a nutrient source. From this initial location, hyphae were observed to spread to adjacent, viable eggs, which were rapidly killed. Multiple foci of invasion within the egg mass sometimes occurred, and could result in the entire egg mass being enveloped by fungi (see Chapter 6).

Laboratory experiments suggested the rate of fungal growth and expansion from egg to egg is likely to be dependent on the species of the fungus and influenced by the thermal and hydric conditions of the nest (see Chapter 9). Although *F. oxysporum* had the fastest linear growth rate under all conditions tested, *F. solani* and *P. boydii* were the most common species of fungi isolated from sea turtle nests. Temporal variation in the prevalent fungi at Heron Is. between successive years may have been the result of different growth preferences. *F. solani* was more prevalent in the 1996/97 nesting season when the ambient temperature was significantly less than in the 1997/98 season when *P. boydii* was more common (see Chapter 4). In the laboratory, fungal colonisation of eggs by *F. solani* was significantly less at 30°C

than at 26 °C and 28°C while *P. boydii* showed greater growth at 30°C than 26°C or 28 °C (see Chapter 9).

F. oxysporum, *F. solani* and *P. boydii* are all common, cosmopolitan soil saprophytes, so their most likely source is the nest substrate. However, *F. oxysporum* and *F. solani* were also isolated from the cloaca of and/or oviduct of nesting and internesting green turtles. This provides some evidence of the potential for acute, intra-seasonal oviductal infection of eggs after mycobiota are accumulated within the cloaca when the ovipositor contacts the substrate during nesting behaviour. Fungus spores could be transported and maintained within the oviduct by the same mechanisms that allow for the transport and survival of intra- and inter-species spermatozoa. The low occurrence of fungi known as egg infectants in the cloacas of nesting turtles (15%) and those up to 12 months post-nesting (0%) suggests the potential for chronic, inter-seasonal oviductal contamination is low (see Section 5.1).

The direct cause of embryo mortality after the sea turtle egg was invaded by fungi was not established to the rigour of Koch's Postulates. However, it was demonstrated that reduction of the respiratory surface area, which might be caused by a dense hyphal mat on the egg exterior, resulted in embryo death (see Section 7.1). Egg mortality was affected by the size and location of the impeded area and the turtle species. Inhibition of the embryonal or respiratory area in 9 day old embryos reduced survival to 0-40% in flatback turtles, depending on the size of the area, and 0% in green turtles, independent of the area covered. Inhibition of the non-respiratory area reduced embryo survival had less effect on survival (80-100%) in

both turtle species. There was 0% survival of all eggs where the entire surface was inhibited. These results suggest that embryo mortality will be greater if the northern hemisphere or embryonal area of the egg is covered by fungus so that gas exchange is significantly reduced, and there may be differences in turtle species susceptibility as determined by weight and/or stage specific metabolic demands of the embryo.

Fungal invasion of the sea turtle egg may also result in calcium loss from the eggshell (see Section 7.2), leading to disruption of eggshell structure and depriving the embryo of calcium required for development. The growth of *F. solani* on the exterior of green turtle eggs significantly reduced the calcium content of the outer, inorganic portion of the eggshell. Consequently, an insufficiency of eggshell calcium during the second half of incubation, when osteogenic demand is maximal, may result in embryo teratogenesis and/or embryo mortality. However, egg failure soon after fungal colonisation (see Chapters 6 and 10) suggests calcium deprivation is unlikely to be the cause of embryo mortality.

F. oxysporum, *F. solani* and *P. boydii* all produce metabolites that would allow them not only to degrade the inorganic portion of sea turtle eggshell to access calcium (organic acids) but also to penetrate the organic portion of the eggshell and invade embryonic tissue (amylase, collagenase, elastinase, lipase and protease) (see Section 7.3). Fungi were isolated from green and loggerhead turtle embryos, but since embryonic death seemed to occur almost simultaneously with fungal presence on the egg exterior in artificial incubation experiments, penetration of the eggshell and utilisation of the embryo and egg contents probably occurs post-mortem. Fungi could then harvest these nutrients and expand to adjacent eggs.

Although it was not demonstrated, there is the potential for mycotoxicosis to have occurred in embryos where hyphae grew on the egg exterior. If fungal metabolites were toxic for sea turtle embryos, their effective range would seem to be limited as adjacent eggs with no external fungus were unaffected (see Section 7.4).

Since egg failure is the event that allows fungal colonisation of the egg mass, environmental factors resulting in embryo mortality are of paramount importance. Hatch success varied significantly with nest depth, substrate conductivity (see Chapter 8) and substrate water content when extreme conditions were imposed on eggs (Chapter 9) but not within *in situ* nests in Chapter 8. Of these factors, substrate conductivity was determined to be the most important (see Chapter 8).

Nest conductivity should not directly influence the fungal colonisation of failed eggs (see Chapter 8) but incubation temperature will (see Chapter 9). The percentage of the entire clutch that was invaded by fungi varied significantly with substrate conductivity (as a function of decreased hatch success) (see Chapter 8). Clutches were not incubated at different temperatures to determine the influence of nest temperature on fungal invasion, but cooler nests may experience a less immediate invasion of failed eggs and a slower linear growth rate of fungi than warmer nests.

Studies of scalation patterns suggest that hatchlings emerging from nests that have a high percentage of failed eggs colonised by fungi should have a similar fitness to those from nests without fungi (see Chapter 12). There was no evidence of fungal invasion of sea turtle nests causing hatchling malnutrition (see Section 7.5).

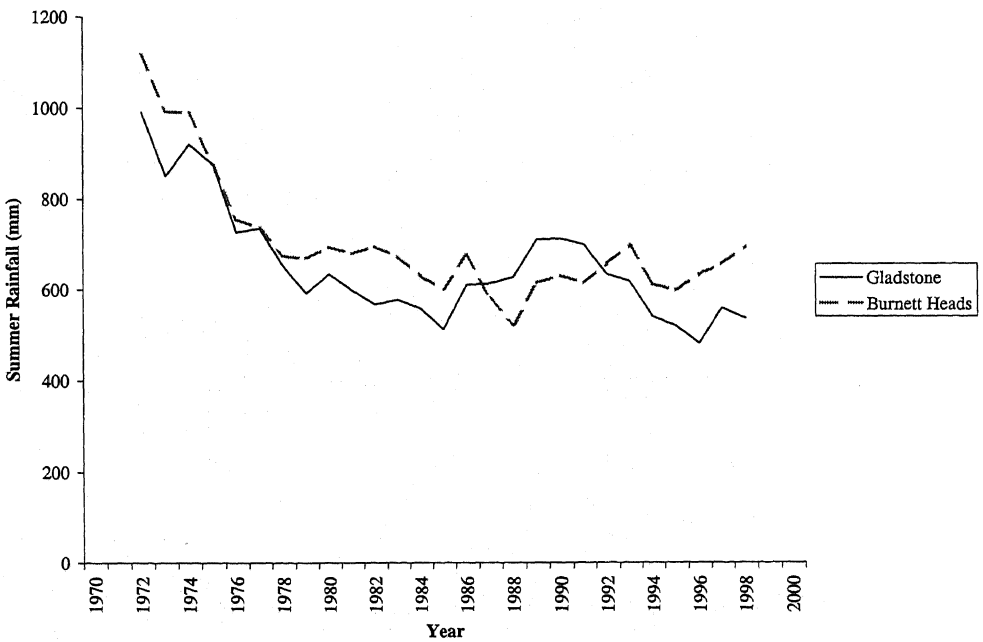
Loggerhead turtle nests at Heron Is. and Wreck Is. were more prone to fungal invasion than green turtle nests at the same rookeries or loggerhead turtle nests at Mon Repos. Increased susceptibility was not due to differences in anti-fungal properties of mucus secreted during oviposition, egg albumen or eggshell ultra-structure. Instead, loggerhead eggs are smaller than those of green turtles with a corresponding higher surface area to volume ratio that would allow greater impact of any particular level of osmotic stress. Consequently in nests of loggerhead eggs at Heron Is. or Wreck Is., where substrate conductivity is high and subsequently water potential is low, embryo mortality rate is likely to be higher than that of adjacent green turtle nests. Loggerhead turtle nests at Mon Repos do not experience a relatively high substrate conductivity.

Elevated egg mortality would result in more potential foci for commencement of fungal invasion(s) of the egg mass. Because of their small size, an equivalent hyphal linear growth rate on loggerhead turtle eggs would allow a greater surface area of any egg to be covered and faster access to adjacent eggs than green turtle eggs. This would result in a lower hatch success of loggerhead turtle nests when compared to green turtles, but not affect the percentage of failed eggs colonised by fungi between species at Heron Is. and Wreck Is. (see Chapter 3). However, hatch success could be expected to vary significantly between loggerhead turtle rookeries, as observed between Mon Repos and Heron Is./Wreck Is. Low substrate mycobiota density at Mon Repos (see Chapter 8) probably resulted in significantly fewer failed eggs being colonised by fungi at this rookery than at Heron Is. and Wreck Is. (see Chapter 3).

If high substrate conductivity results in higher egg mortality and the increased availability of failed eggs to act as nutrient foci for invasion of sea turtle nests, long term rainfall patterns are of great interest. Mon Repos is the largest loggerhead turtle rookery in the southern Pacific and, currently, does not demonstrate a low hatch success or high fungal invasion of turtle nests. However, C.J. Limpus (pers.comm.) has observed an increasing occurrence of fungi on failed eggs. This corresponds with decreasing summer (September to March) rainfall at Burnett Heads (the nearest permanent weather station to Mon Repos- see Figure 13.1) to that approaching Gladstone's (the nearest permanent weather station to Heron Is.- see Figure 13.2) and a potentially increasing substrate conductivity at Mon Repos as salts are not leached from the nest substrate. With the rapidly decreasing numbers of nesting loggerhead turtles in eastern Australia and a potential increase in unfavourable nest conditions at the largest remaining rookery, further research is required to substantiate the role of nest conductivity in egg mortality, its possible relationship with substrate conductivity, and the potential role of rainfall in maintaining an optimal nest environment.

Figure 13.1 Burnett Heads 5 Year Running Average Summer Rainfall.

Figure 13.2 Burnett Heads and Gladstone 5 Year Running Average Summer Rainfall.



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APPENDIX A

MICROBIOLOGICAL MEDIA

Recipes for the most common media used during culture of fungi are given below. Unless otherwise stated, recipes were obtained from Atlas (1993). Media was made in 1L lots and allocated into 500mL Schott bottles for heat sterilisation at 15psi and 121°C for 15mins, then cooled to 55°C prior to preparation of plates in a Biological Safety Cabinet Class II. Plates were stored at 3°C until required.

Half-strength Potato Dextrose Agar (HPDA)

20g PDA

8g agar

1L distilled water

Carnation Leaf Agar (CLA)

Young carnation leaves were blanched with boiling water then embedded (3 leaves per plate) in a plate of WA prior to its complete solidification. CLA was recommended for use by Seifert (1996) but no recipe could be found. Subsequently, this recipe was developed by A.D. Phillott.

Malachite Green Agar (MGA)

NSMB with 2.5 ppm of Malachite Green

(Castellá *et al.* 1997)

Nash and Snyder Medium Base (NSMB)

15g peptone

1g $KH_2.PO_4$

0.5g $MgSO_4.7H_2O$

20g agar

1L distilled water

(Castellá *et al.* 1997)

Potato Dextrose Agar (PDA)

40g PDA

1L distilled water

(BBL commercial preparation)

Water Agar (WA)

15g agar

1L distilled water

Water Potential Media

Water potential basal medium was made according to the following recipe:

0.725g Na_2HPO_4

0.725g KH_2PO_4

0.120g $MgSO_4$

0.100g NaCl

0.400g NH_4NO_3

1.800g glucose

1.100g	yeast extract
1.000g	malt extract
1L	distilled water

Prepare 10 flasks each with 100mL of water potential basal medium and 1.5g agar, and one of the following:

Flask	KCl (g)	molarity KCl	ψ (–kPa)
1	0.0000	0.0	173.23
2	0.7455	0.1	467.80
3	1.4910	0.2	921.60
4	2.2365	0.3	1372.20
5	2.9820	0.4	1820.90
6	4.4730	0.5	2268.60
7	5.9640	0.6	2716.20
8	7.4550	0.7	3168.90

Each flask produces 5 plates of media. It is important to prevent the formation of condensation: media should be cooled to 55°C before pouring, and plates incubated in sealed bags or containers. Handle carefully so as not to dislodge any water droplets that may form.

APPENDIX B

DEVELOPMENT OF LIFE HISTORY TABLES FOR GREEN, LOGGERHEAD, HAWKSBILL AND FLATBACK EMBRYOS AT ROOKERIES NOT AFFECTED BY FUNGI.

To develop life history tables for sea turtle embryos, natural mortality of *in situ* eggs was determined by examining rookeries not adversely affected by fungi. Heron Is. and Wreck Is. were, therefore, excluded from this analysis as fungal colonisation of turtle nests occurring at these islands was regarded as an extrinsic cause of embryo mortality. During the excavation of emerged turtle nests (green and hawksbill at Milman Is., loggerhead at Mon Repos and flatback at Peak Is.) in the 1998/99 nesting season, unhatched eggs were opened and their contents examined to determine embryonic stage at death. Embryos were staged by macroscopic examination into categories following Miller (1985):

Stages 6-10	nil embryonic development apparent.
Stages 11-16	embryonic development evident only by traces of blood resulting from decomposition of extra-embryonic membranes.
Stages 17-25	limbs and carapace developing.
Stages 26-28	embryo pigmented.
Stage 29	embryo approximately the size of a full-term hatchling.
Stage 30	eggshell pipped.

Determining the number of eggs dead in each category allowed the development of life history tables and mortality curves following the procedures of Brower *et al.* (1997) and Ricklefs and Miller (1999). It was assumed that the number of viable

embryos at Stages 6-10 was equal to the number of eggs laid that were not later predated, as there is little evidence for infertile eggs. Thereafter, the number of embryos entering each category (l_x) was calculated as the number of embryos surviving the previous embryonic stages:

$$l_x = l_{x-1} - d_{x-1}$$

where d_x was the number of embryos counted as dead for each category. The stage-specific mortality rate (m_x) could then be calculated as

$$m_x = d_x / l_x$$

Life tables were constructed for green and hawksbill turtle embryos at Milman Is., loggerhead embryos at Mon Repos and flatback embryos at Peak Is. (see Tables B.1-B.4). The mortality rate for each species is presented in Figure B.1.

Natural mortality was highest immediately after oviposition at Stages 6-10. The remainder of incubation of all 4 species showed a low, constant stage-specific mortality (typically <1%) with one exception. It is unknown if the elevated flatback mortality at Stages 17-25 and Stages 26-28 was a result of environmental factors occurring on the beach during the 1998/99 nesting season (e.g. spring tides) or whether it represents the embryos increased vulnerability at this stage, possibly to the dry conditions present at Peak Is. (see Hewavisenthi 1999). Apart from this exception, the mortality rate for each species followed a similar pattern (see Figure B.1) so it is assumed that in the absence of abnormal external factors, this natural mortality trend may be applied to all sea turtle embryos.

Table B.1 Life table showing mortality of green turtle embryos from 7 nests at Milman Is. in the 1998/99 nesting season.

Embryonic Stage	# Embryos Entering Embryonic Stage (l_x)	# Embryos Dying (d_x) in Stage	Mortality Rate (m_x) in Stage
Stage 6-10	658	39	0.0593
Stage 11-16	619	4	0.0065
Stage 17-25	615	5	0.0081
Stage 26-28	610	9	0.0148
Stage 29	601	5	0.0083
Stage 30	596	2	0.0034

Table B.2 Life table showing mortality of hawksbill turtle embryos from 32 nests at Milman Is. in the 1998/99 nesting season.

Embryonic Stage	# Embryos Entering Embryonic Stage (l_x)	# Embryos Dying (d_x) in Stage	Mortality Rate (m_x) in Stage
Stage 6-10	3642	340	0.0934
Stage 11-16	3302	3	0.0009
Stage 17-25	3299	38	0.0115
Stage 26-28	3261	19	0.0058
Stage 29	3242	36	0.0111
Stage 30	3206	11	0.0034

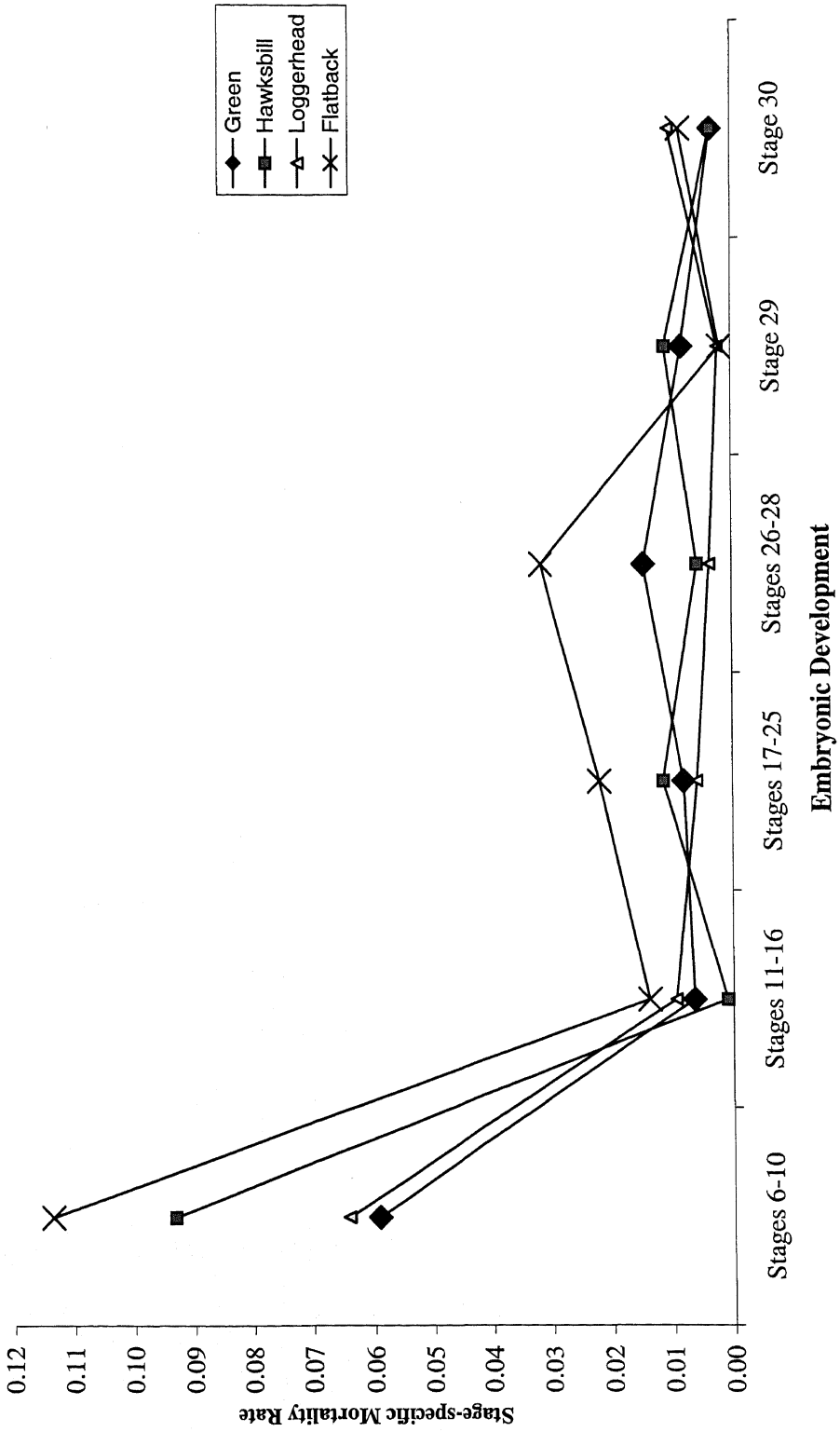
Table B.3 Life table showing mortality of loggerhead turtle embryos from 12 nests at Mon Repos in the 1998/99 nesting season.

Embryonic Stage	# Embryos Entering Embryonic Stage (l_x)	# Embryos Dying (d_x) in Stage	Mortality Rate (m_x) in Stage
Stage 6-10	1462	94	0.0643
Stage 11-16	1368	13	0.0095
Stage 17-25	1355	8	0.0059
Stage 26-28	1347	5	0.0037
Stage 29	1342	3	0.0022
Stage 30	1339	14	0.0105

Table B.4 Life table showing mortality of flatback turtle embryos from 44 nests at Peak Is. in the 1998/99 nesting season.

Embryonic Stage	# Embryos Entering Embryonic Stage (l_x)	# Embryos Dying (d_x) in Stage	Mortality Rate (m_x) in Stage
Stage 6-10	2524	287	0.1137
Stage 11-16	2237	31	0.0139
Stage 17-25	2206	49	0.0222
Stage 26-28	2157	69	0.0320
Stage 29	2088	4	0.0019
Stage 30	2084	18	0.0086

Figure B.1 Mortality of Green, Hawksbill, Loggerhead and Flatback Sea Turtle Embryos



APPENDIX C

MINIMISING FUNGAL INVASION DURING THE ARTIFICIAL INCUBATION OF SEA TURTLE EGGS

The artificial incubation of sea turtle eggs has become increasingly common for research and conservation purposes. It usually involves the collection of eggs, transport to a laboratory (potentially long distance), then incubation within a container and/or incubator on sand or an artificial substrate such as vermiculite. During incubation it may be necessary to inspect eggs to monitor development and mortality, and maintain moisture conditions.

At all stages during these procedures, eggs are exposed to infectants which have the potential to kill a proportion, or all, of the eggs. To minimise egg mortality, precautions can be taken to reduce egg exposure to microbiota.

EGG COLLECTION

Collecting eggs directly from the ovipositor minimises their exposure to fungal spores dispersed during the turtle's body-pitting and egg-chambering. Eggs may be caught by a gloved hand placed in the rear of the egg chamber (part of the chamber may need to be widened). Though some species are more tolerant than others, care must be taken not to disturb the turtle by use of excessive light, or by touching the ovipositor and hind flippers. If eggs cannot be collected during oviposition, they may be excavated after laying has concluded, but this method increases contact with soil microbiota. Some workers have attempted to place a collection bag in the egg chamber

during oviposition. This often disturbs the turtle, resulting in nest abandonment, collapse of the egg chamber during bag insertion, and difficulty removing it when full. This method may be more successful with species that do not dig a deep body pit and/or on beaches with relatively moist sand, where the rear of the chamber may be excavated completely for easier insertion and retrieval.

Eggs should be placed directly into sterile bags; autoclave disposal bags (e.g. Sarstedt®) are recommended due to their strength. Placing the bag inside a bucket helps support the weight of the eggs and prevents weakening and tearing of the bag under stress. The neck of the bag should be twisted and folded over before securing. Prior to transport or incubation, eggs may be washed in sterile distilled water or a solution such as Aricide® (Hibberd 1996) to remove microbes from the egg exterior. However, washing removes the cloacal mucus and its potential anti-fungal properties (see Chapter 11)). After washing, the exterior of the egg should be patted dry using a clean disposable cloth to remove excess water and prevent ice-crystal formation and disruption of the shell structure during low-temperature transport.

EGG TRANSPORT

Eggs may be transported long distances by following the procedures of Harry and Limpus (1989). Eggs depressed to 7-10°C (within 2hrs of oviposition) may be held for 48hrs, allowing collection of multiple clutches over several nights and subsequent transport. It is recommended that eggs remain in the collection bags during this time to minimise exposure to microbes. The bags should be arranged so that they are stable, and air spaces filled with clean, expanded polystyrene pellets for support and insulation.

EGG HANDLING

To minimise movement induced mortality, egg orientation should be maintained (Limpus *et al.* 1979, Parmenter 1980). Single-use sterile gloves should be worn, and eggs handled in a room or area with minimal air flow or disturbance. If the area is to be used permanently for incubation purposes, a dual door system with an intermediate isolation area minimises air disturbance during entry and exit. Workers must wait in the isolation area until the first door has completely closed before opening the second.

EGG INCUBATION

Prior to egg collection, incubators should be cleaned with a 5% sodium hypochlorite bleach solution, then rinsed with a 5% solution of disinfectant. Incubation containers (which will hold the eggs) may be sterilised by autoclaving at 121°C for 15mins with the mouth covered in aluminium foil, or with a 5% bleach solution followed by a sterile water rinse.

If incubating on sand, it should be collected from areas relatively free of organic material. Sand may be sterilised by autoclaving in small lots. Thermal sentinels (e.g. Thermalog®S strips) should be used to ensure effective heat sterilisation at the core. These checks can be ceased when the performance of particular autoclaves, sand types, sand quantities etc. is quantified.

Labels on cardboard or other biodegradable material should not be placed in or on the substrate during incubation as they provide nutrient sources for mycobiota. Instead, the exterior of the container should be labelled. The required moisture conditions are

maintained by adding sterile water either from clean spray bottles, or by use of a sterile water, sub-surface trickle irrigator. If the plastic tubing from the latter is to be re-used, it should be first be cleaned with a commercial algaecide (used in cleaning swimming pools) then rinsed with sterile water.

When removing eggs (to weigh, measure, candle etc.) wear sterile, single-use gloves and ensure the equipment is clean and that air flow around the area is minimal. If eggs are to be weighed, sand can be removed using a soft brush that will not damage the eggshell. Using a cloth to remove sand has the potential to drag sand across the egg surface, disrupting structural integrity of the eggshell. Eggs that fail to develop a white spot, that show signs of yellowing, or have fungal growth on the exterior should be removed. Mass egg mortality often follows fungal contamination of a single moribund egg as hyphae spread to adjacent viable eggs (see Chapter 6). This potential spread can be minimised by ensuring eggs are not in contact with each other, though this is not always practicable.

In the event of egg invasion, the species of fungus can often suggest the source of the contamination. *F. oxysporum*, *F. solani* and *P. boydii* are regularly isolated as soil-borne pathogens on the exterior of failed eggs (see Chapter 4)). In contrast, *Aspergillus* spp. have been contracted by air-borne contamination (pers.ob.). Identification of the fungus may lead to its point of source, allowing protocols to be modified so as to eliminate contamination.