STUDIES OF MYCOSES IN FARMED ESTUARINE CROCODILES, (*Crocodylus porosus* Schneider 1801).

A thesis submitted by

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DECLARATION

This thesis contains no material which has previously been submitted for a degree or diploma to any other university or institution of tertiary education. The work described herein, except where otherwise acknowledged, was carried out by me during the period of my candidature.

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Plate 1: The author with juvenile *C. porosus* (photo S. Lever).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

ABSTRACT

The ubiquitous fungus, *Fusarium solani* (Mart.) Sacc., (teleomorph *Nectria haematococca* Berk and Broome) was frequently isolated, both superficially and systemically, from diseased juvenile farmed crocodiles, *Crocodylus porosus* Schneider 1801. At autopsy, various internal tissues showed granulomatous inflammations from which the same fungal pathogen was readily isolated. Asymptomatic tissues were also shown to be infected. Other fungi isolated with less frequency from the animals were *Paecilomyces lilacinus, Cladosporium* sp. and *Aspergillus* sp.

Infection rates at a commercial crocodile farm had reached epidemic proportions with mortality and morbidity in excess of 50% of each year's eggs and 50% of each year's hatchlings. Environmental samples showed that the pathogen was widely distributed in the farm environment. Contamination of freshly laid eggs by the pathogen was determined as the probable primary cause of infection, along with subsequent physical trauma in juveniles. Natural nesting material was implicated as a major source of egg contamination. To prevent infection of eggs, changes to procedures in the artificial incubation techniques used at the farm were carried out and a significant increase in hatchability was achieved.

Treatments of diseased animals met with varying degrees of success. Attempts were made to control the incidence of the disease by modifying husbandry practices after investigation of environmental parameters. Hatchling survival was significantly improved.

Standard autopsy, histological and mycological procedures were used to isolate and identify the pathogens. Various environmental monitoring methods were employed with a view to minimising stress on the animals. Scanning electron microscopy was used to determine the method of access by the fungi into the eggs.

1. INTRODUCTION

1.1 Aim

The initial aims of this project were

- to investigate the epidemiology of systemic mycotic disease in juvenile farmed estuarine crocodiles, *Crocodylus porosus* Schneider 1801;
- to describe the histopathology of the infection;
- to ascertain the environmental factors which contributed to or directly caused the infection;
- to determine the most likely variables which could be causally associated with increased infectivity; and
- to attempt treatment of the diseased animals and subsequently reduce the high hatchling and yearling mortality.

The project was later expanded to investigate fungal presence in the eggshell and eggs of farmed *C. porosus*, and to reduce the incidence of embryo mortality due to fungal infection thereby increasing the number of healthy live hatchlings.

1.2 Significance

The research reported here carries an economic significance to what is still a fledgling Australian industry. Major losses of juveniles cause financial problems for crocodile farmers (Lever, pers. comm.). Moreover, high losses of fertile viable eggs during incubation, combined with hatchling and yearling mortality may be financially ruinous. There is a known direct monetary loss from the high costs of artificial incubation of eggs, and the daily costs of feeding, cleaning and housing animals which subsequently die (Lever, pers. comm.). Indirect losses are due to failure to reach skinning size, reduction in quality and value of skins, and contamination of a source of gourmet meat. There is also a higher cost in maintaining the surviving slow growers (runt syndrome). Diseased animals are not suitable for public display and are, therefore, detrimental to the tourism industry. If the number of healthy animals decreases substantially, this may result in fewer tourists, a reduced restaurant and kiosk trade and fewer farm staff. Eventually employment in both the tourism and crocodile industries may suffer. Conservation of this endangered species may also be

affected as farmed crocodiles are a potential source of restocking depleted natural populations in Australia.

1.2.1 Australian Crocodile Industry

In 1991, there were 2532 *C. porosus* skins produced in Australia (Luxmoore, 1994). Production in 1992 rose to 4405 skins, and in 1993 to 7063 (Collins, 1995) with *C. porosus* skins accounting for around 10% of world trade in all crocodilian skins (Onions, 1991). Australia produced less than 1% of the world supply of all crocodilian skins in 1988 (Webb, 1988 cited in McKelvie and Treadwell, 1991).

Quality *C. porosus* skins are readily marketed in the size 34-35 cm belly width (animal length 1.5 m), and for larger skins, a belly width of 40 cm plus (Onions, 1991). On average it takes two to three years to reach the preferred size of 1.5 m, with up to five years for some animals in captivity (McKelvie and Treadwell, 1991). A 1.5 m crocodile will yield on average 7-10 kg of flesh with around 1% fat.

As reported by King (1990) prices paid (in U.S. dollars) to farmers in Queensland for *C. porosus* in November 1989 were:

- wet salted belly hides, US$9.00 to US$9.50 per cm width;
- frozen tail, leg, and jaw muscle meat, US$20.00 per kg;
- frozen backstrap meat, US$25.00 per kg.

In 1990/91 the estimated value of total skin production, meat, and other crocodile products from both *C. porosus* and *C. johnstoni,* the Australian freshwater crocodile, was Aus$2.75M. Tourism benefited tourist-orientated farms a further estimated income in excess of Aus$2.5M (Onions, 1991). It was estimated that the industry had the potential to rise to Aus$15M by 1995 from Northern Territory production alone, with employment to rise by approximately 700% in the same period on Northern Territory farms. Over 90% of crocodile skins produced in Australia are exported, with significant benefits achieved by value adding to raw skins, *eg.* tanning and finishing adds 60-70%, manufacturing adds 200%, retailing crocodile products adds 300%, with designer label products much higher again (Onions, 1991). The skin of *C. porosus* is considered the finest crocodilian skin available thus maintaining the price premium. (McKelvie and Treadwell, 1991). In their investment analysis of crocodile farming, McKelvie and Treadwell (1991) calculated an income of $550 per estuarine

crocodile at harvestable size with a range of $500 to $600 (average age 3.5 years, range 2 to 5 years, belly width 34-35 cm). By reducing the harvest age to 2.5 years (range of 1.5 to 3.5 years), the likely returns increased substantially to almost double the base case when maintaining a 50:50 mix of *C. porosus* and *C. johnstoni*. Due to the difference in skin values and growth rates between these two species, the return for a farm producing only *C. porosus* would be further increased.

1.2.2 Classification and World Distribution

The classification of Saltwater or Estuarine Crocodiles (Webb & Manolis, 1988) is:

Phylum:	Vertebrata
Class:	Reptilia
Sub-Class:	Archosauria
Order:	Crocodilia
Sub-Order:	Eusuchia
Family:	Crocodylidae
Sub-Family:	Crocodylinae
Genus:	*Crocodylus*
Species:	*porosus*

Modern day crocodilians all belong to the sub-order Eusuchia, which has a single family Crocodylidae (Bellairs, 1987). This family has three sub-families, the Crocodylinae (with two genera *Crocodylus* and *Osteolaemus*), the Alligatorinae (with four genera *Alligator*, *Caiman*, *Paleosuchus*, and *Melanosuchus*) and the Gavialinae (genera *Gavialis* and possibly *Tomistoma*, previously regarded as belonging to Crocodylinae). There are twenty seven species and sub-species in the family Crocodylidae. *C. porosus* is currently listed under both Appendices I and II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is recognised as *endangered* by the International Union for Conservation of Nature and Natural Resources (IUCN) (Groombridge, 1987).

C. porosus is a widespread species which ranges from eastern India and Sri Lanka, through southeast Asia to the Philippines, through Malaysia, Indonesia, Papua New Guinea and east to the Solomon Islands and Vanuatu, and south to northern Australia. Populations vary from severely depleted, to rare, to declining, in most of their natural

range. However, adequate wild population levels are maintained in Papua New Guinea and northern Australia. Estuarine crocodiles usually inhabit coastal mangrove swamps or tidal river systems but also live in freshwater rivers and swamps. They have also been recorded at sea and on land in areas beyond their normal breeding range. They have been categorised as *endangered* because of habitat destruction and former extensive hunting for their skins by humans. Trading in skins and other products is currently facilitated by the rearing of hatchlings in captivity from wild-collected young (ranching), or by captive breeding (farming). This allows conservation of wild populations by decreasing indiscriminate hunting (Groombridge, 1987).

1.2.3 Survival and Growth

Estuarine crocodiles mate and nest during the summer monsoon season, typically October to February, but sometimes as late as May. An average of fifty eggs are incubated in a nest which is constructed of soil and vegetation by the female. In the wild, on average 80% of eggs die before hatching and a further 50% of these hatchlings die within the first year. A further 25% die each year between one and three years of age, and annual losses between ages three to five years may be around 50 to 60%. This results in an overall 1 to 2% survival from egg to five years of age in the wild (Webb and Manolis, 1988; Webb and Manolis, 1989).

The average weight of wild crocodiles at hatching is 0.072 kg and average total body length is 29.3 cm. At age one year these have increased to 0.870 kg and 73.0 cm, and at age four years, to 10.6 kg and 154.5 cm. (Webb and Manolis, 1989). In captivity the size and weight for age can be greatly increased with animals around 20% heavier than wild crocodiles. Males generally grow faster than females. Farm bred males mature at around 3 m, females at 2.1 m, both at age six to seven years. In the wild, maturity would be reached at 2 to 2.3 m and age twelve years for females, and 3.35 m and age sixteen years for males (Webb and Manolis, 1989).

1.3 Historical Background

There are fifteen crocodile farms in Australia, six in Queensland, six in the Northern Territory, and three in Western Australia. Some are privately owned. Others are

Government sponsored Aboriginal Co-operatives. Some are tourist orientated whereas others are not open to the general public. Some operate captive breeding programs, and others are licensed to collect crocodile eggs from the wild for hatching and rearing in the farm situation. Koorana is a family owned and operated commercial farm with a full captive breeding program. Some income is generated from the tourist market by way of day time guided tours, a licensed restaurant, and a souvenir kiosk. Koorana-derived crocodile meat for human consumption is available in the farm restaurant, and crocodile skin products such as handbags, belts, boots, and wallets are for sale in the kiosk. Skeletal preparations, eggs and skins are also for sale. Night spotlight and dinner tours are available. Educational tours for school groups are conducted regularly as are visits to schools by the Koorana Education Officer. Crocodiles and their handlers are available for hire. A Crocodile Investment Lease program is in effect in which investor's lease a female crocodile and gain profit by the sale of the progeny. A future development is the construction of elevated accommodation units inside an enclosure housing the farm's third lake. The bungalows will be marketed towards international tourists, who will be able to look out over the crocodiles in the lake. The complex has been the recipient of several tourism awards and makes an overall significant contribution to the local economy. In addition to family members, there are several casual, part-time and full time employees.

The land for the Koorana Crocodile Farm at Coowonga, approximately forty kilometres east of Rockhampton, was purchased in 1980. It consists of one hundred and thirteen hectares of melaleuca and eucalyptus open woodland, mangroves, mud flats and salt pans. The area was considered to be ideal estuarine crocodile territory. It is bordered on three sides by Coorooman, Cawarral and Horton Creeks, which are tidal and also by Coowonga Road. During four metre tides the farm becomes an island (known locally as Savage's Island), with causeway access. Salt water pumped from the nearby tidal creeks at high tide is used to fill lakes for the adult and sub-adult crocodiles. An aerial view of Koorana Crocodile Farm is presented in Plate 2a.

The farm is situated approximately on the Tropic of Capricorn, latitude 23°20' south and longitude 150°45' east, and is the most southerly crocodile breeding farm in Australia. It is over four hundred kilometres south of the nearest crocodile farm at

Mackay, Central Queensland and is on the southern extremity of the saltwater crocodiles' natural east coast range (refer Figure 1). Sightings of adult estuarine crocodiles are not uncommon in the saltwater city reaches of the Fitzroy River on which Rockhampton is located. Crocodiles are also seen in the freshwater reaches for several kilometres upstream of the Fitzroy Barrage.

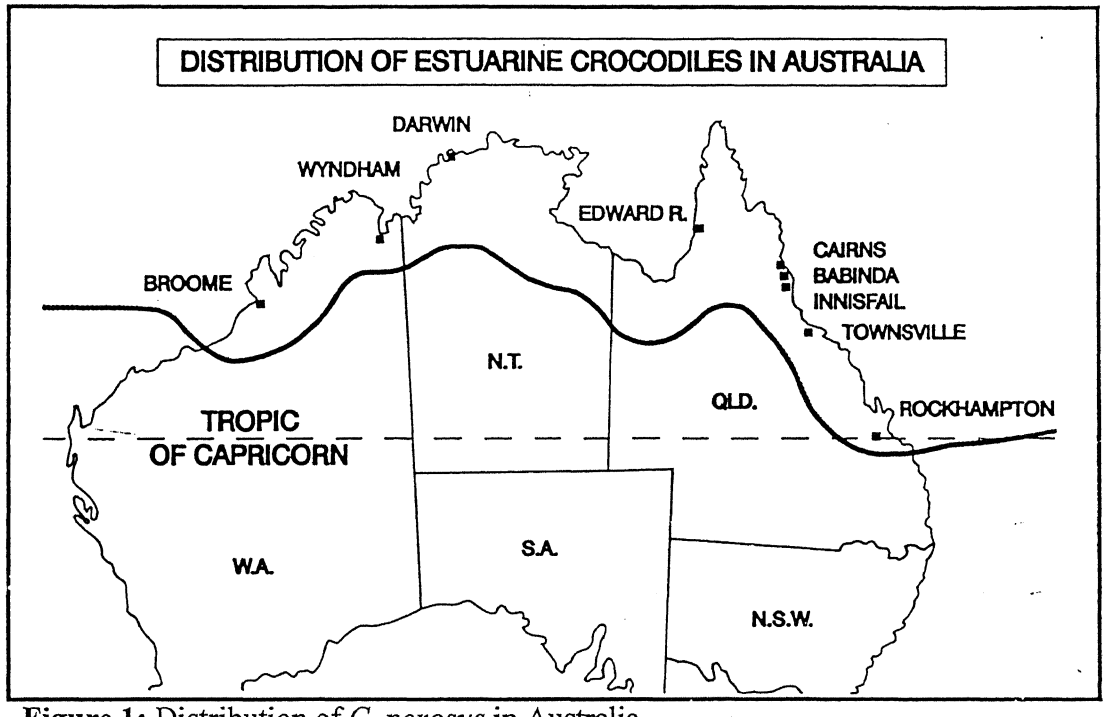

Figure 1: Distribution of *C. porosus* in Australia.

Winters at the experimental location can be quite cold (range from 5°C to 25°C). Thus, a late summer wet season, resulting in late nesting and hatching, would lead to very high hatchling mortality in the wild due to cold stress. At Koorana, hatchlings and juveniles up to two years and sometimes older must, therefore, by necessity be kept indoors in a heated environment. In winter months, older animals (three to four years old) are also provided with heated sheds which straddle outdoor artificial ponds. The egg incubation facilities, hatchling and yearling buildings, and grow-out lakes are located away from public areas. However, some hatchlings are housed in a heated chamber which can be viewed from inside the restaurant at the farm. Adult breeding crocodiles are housed in three lakes accessible to tourists via protected raised walkways. As a tourist attraction, crocodiles are fed daily from a covered platform above the main lake, pictured in Plate 2b.

Plate 2a: Aerial view of Koorana Crocodile Farm (photo J. Lever).

Plate 2b: Daily feeding of adult crocodiles to entertain and educate tourists.

Several large breeding pairs of crocodiles are located in separate smaller enclosures, one pair to each enclosure. These are animals four to six metres in length, with individual names and a 'capture story' for each.

The farm was officially opened in 1981 with nine crocodiles. The next few years were involved in hunting wild rogue crocodiles for breeding stock, building and setting up facilities including the licensed restaurant and souvenir kiosk, giving educational tours, and receiving tourists. Captive breeding commenced in 1984. Eggs laid in October 1985 and which hatched in early 1986 succumbed to high early hatchling mortality. Pathological investigations were carried out at that time by Hunt and Aiken (1986). Nothing was obvious at post-mortem and no histological abnormalities were noted.

In 1987 there was an increase in hatchling numbers and also in subsequent unexplained deaths. In 1988 there was a further increase in hatchling mortality. Work by Hunt *et al* (1988) revealed a systemic mycoses, with the causal agent determined as a species of *Fusarium*. This was later re-isolated by Harrower (1988) and identified as *Fusarium solani* (Mart.) Sacc. This was confirmed by J. W. Rippon and also by Dr. Ailsa Hocking of CSIRO Division of Food Research in Sydney. Subsequent re-examination of the 1986 tissues using a specific fungal stain also showed evidence of invasive fungal hyphae. In 1989 further deaths of hatchlings occurred. In April and May of 1990 losses of both hatchling and yearling stock commenced. This mortality peaked in September of that year in epidemic proportions with up to nine animals dying daily of the infection. In excess of fifty percent of each year's hatchlings died (Lever, pers. comm.).

Treatments and preventative measures used up to 1990 had involved spraying pens with commercial plant fungicide, sealing pens with antifungal paint, feeding potassium iodide in the food, alternate daily use of freshwater and seawater in the pens, and daily scrubbing of the pens with commercial bleaches (Lever, pers. comm.). None of the treatments prevented the progress of the epidemic. This work commenced as a part-time project in early 1990.

1.4 Literature Survey

1.4.1 *Fusarium*, diseases and reptilian mycoses

Members of the fungal genus *Fusarium* are ubiquitous moulds which are not normally pathogenic to man or animals (Rippon, 1988). They are generally considered to be opportunistic pathogens. They have been found by various authors in beach sand, and fertile cultivated, rangeland and forest soils. They may be found in both fresh and salt water. They are common in subterranean and aerial plant parts, plant debris and in other organic substrates. They have a broad geographic range (tropical, temperate, desert, alpine and arctic areas). Conidia of the fungus may be windborne, perhaps after an initial 'rain splash' event to project the spores into the moving air mass. Hatai and Egusa (1978), cultured *Fusarium* in Sabouraud dextrose agar at up to 10% sodium chloride concentration, and in Sabouraud dextrose broths varying in pH from four to eleven. The study of *Fusarium* has been the subject of many books (Booth, 1971a; Toussoun and Nelson, 1976; Nelson *et al*, 1981, 1983; Moss and Smith, 1984). *Fusarium* diseases are frequently reported in plants and given common names such as wilts, cankers, head-blights and rots (Booth, 1971a, 1984; Price, 1984). Some species are recognised as insect pathogens (Barson 1976; Claydon and Grove, 1984) and others are known to be responsible for mycotoxin production in stored grains (*eg.* corn, wheat, rice), hay and fodder (Ainsworth and Austwick, 1955). When infected grains are ingested by animals or humans, the mycotoxins have deleterious effects (Nelson *et al*, 1983; Drysdale, 1984; Bosch *et al*, 1989; Beri *et al*, 1991) which may be fatal. Bonner (1995) reported work by other researchers investigating deaths of horses in Great Britain, Europe, Australia and Argentina. Toxins, produced by endophytic fungi living in the grasses eaten by the horses, were thought to be the probable cause of death. It was suggested that fungi of the genus *Fusarium* were the aetiological agents. Research into that problem is continuing. Roffe *et al* (1989) reported large-scale natural die-offs (5000 cranes in one event) of wild Sandhill Cranes (*Grus canadensis*) over two consecutive years. The deaths were attributed to fusariomycotoxicosis associated with the ingestion of mouldy peanuts, which were the main diet of the cranes. A toxigenic *Fusarium* sp. was recovered from waste peanuts within the area.

Fusarium species have infected several invertebrate species (refer Table 1), reptiles including crocodilians (refer Table 2), fish, birds, and mammals (refer Table 3), including humans (refer Table 4). *F. solani* has been found in several species of Crustacea (refer Table 1), sea turtles and crocodiles (refer Table 2), sharks, fish, seals and sea-lions (refer Table 3).

F. solani has infected fertile eggs from snakes in captivity, resulting in embryo mortality and an abnormally small hatchling (Kunert *et al*, 1993). *F. solani* was implicated in the deaths of farmed Loggerhead turtle hatchlings after the turtle eggs hatched in contaminated sand (Rebell *et al,* 1971). *Fusarium* sp. was also isolated from wild Loggerhead turtle eggs with high mortality (Gyuris, pers. comm.).

Fungal diseases in reptiles are becoming more frequently reported, the most common being due to species of *Beauveria, Paecilomyces, Aspergillus, Cephalosporium* and *Fusarium.* Fungal diseases have been reported in turtles, tortoises, snakes, lizards, chameleons (refer Tables 2, 5a and 5b) and less frequently in crocodiles and alligators (refer Table 2, and section 1.4.2 to follow).

The majority of the cases listed in Tables 1, 2, 3 and 5 were from animals in captive or farmed conditions. Some exceptions were *F. solani* infections in wild Lobsters (McAleer, 1983 in Connole, 1990), *F. oxysporum* infections in wild Alligator eggs (Schumacher and Cardeilhac, 1990) and *Fusarium* sp. infections in wild Loggerhead turtle eggs (Gyuris, pers. comm.). Treatments where attempted were frequently not successful. Human cases also frequently had high mortality.

Fungal pathogen	Species affected	Reference
Fusarium sp.	Kuruma prawn, *Penaeus japonicus*	Egusa and Ueda, 1972
Fusarium sp.	American lobster, *Homarus americanus*	Lightner and Fontaine, 1975
F. solani	Large Elm Bark Beetle, *Scolytus scolytus*	Barson, 1976
Fusarium sp.	California Brown Shrimp, *P. californiensis*, Brown shrimp, *P. aztecus*, Pink shrimp, *P. setiferus*	Solangi and Lightner, 1976
F. solani	*P. japonicus*	Hatai *et al,* 1978
F. solani	*P. japonicus*	Hatai and Egusa, 1978
F. solani	*H. americanus*	Fisher *et al,* 1978
Fusarium sp.	Freshwater prawn, *Macrobrachium rosenbergii*	Burns *et al,* 1979
F. solani	Lobster, *H. vulgaris*	Alderman, 1981
(a) *F. solani* (b) *Fusarium* sp.	(a) *P. japonicus* (b) Review of *P. aztecus, P. duorarum, P. californiensis*	(a) Bian and Egusa, 1981 (b) citing others
F. solani	Western Rock Lobster, *Panulirus cygnus*	McAleer, 1983 in Connole, 1990
Fusarium spp.	Several species of insects, (review)	Claydon and Grove, 1984
F. solani	*P. californiensis*	Hose *et al,* 1984
F. solani	Crustaceans, (review)	Austwick, 1984
F. tabacinum	Crayfish, *Austropotamobius pallipes*	Alderman and Polglase, 1985
F. solani	Nematode egg masses, *Meloidogyne javanica*	Reddy and Mani, 1988
F. solani	Crayfish, *Astacus leptodactylus, Pacifastacus leniusculus*	Chinain and Vey, 1988
F. solani	Shrimp, *P. semisulcatus*	Colorni, 1989

Table 1: *Fusarium* infections in Invertebrates.

Fungal pathogen	Species affected	Reference
F. solani	Turtle, *Caretta caretta*	Rebell *et al,* 1971
Fusarium sp.	African Dwarf Crocodile, *Osteolaemus tetraspis*	Keymer, 1974
Fusarium sp.	*Caiman crocodilus fuscus*	Kuttin *et al*, 1978
F. solani	Burmese python, *Python molurus bivitattus*	Jacobson, 1980
Fusarium sp.	Caiman	Jacobson, 1980 in Marcus, 1981
F. solani	Green turtles, *Chelonia mydas*	Glazebrook, 1980 in Connole, 1990
F. solani	Turtles, *Lepidochelys kempi, C. mydas, C. caretta*	Rebell, 1981
(a) *F. oxysporum*	(a) Indian Python eggs	(a) Austwick and Keymer, 1981 and citing by review
(b) *F. urticearum*	(b) Lizard, *Lacerta viridis*	(b) Blanchard, 1890
(c) *Fusarium* sp.	(c) Ophidian snake	(c) Grünberg *et al*, 1963
(d) *Fusarium* sp.	(d) Tortoise, *Testudo radiata*	(d) Frank, 1966
(e) *F. oxysporum*	(e) *Chameleo dilepis*	(e) Poelma, 1971
(f) *F. solani*	(f) *T. radiata*	(f) Borst *et al,* 1972
(g) *F. oxysporum*	(g) *Boa constrictor*	(g) Vroege, 1972
(h) *F. oxysporum*	(h) *Epicrates cenchria maurus*	(h) Zwart *et al*, 1973
Fusarium spp.	Several reptile species (review), including crocodilians	Austwick, 1984
F. moniliforme	Alligator, *A. mississippiensis*	Frelier *et al*, 1985
F. solani	Kemp's Ridley eggs, *L. kempi*	Leong, pers. comm. in Wyneken *et al*, 1988
F. oxysporum	Alligator eggs, *A. mississippiensis*	Schumacher and Cardeilhac, 1990
F. solani, *F. equiseti* *F. oxysporum* *F. semitectum*	Estuarine crocodile, *C. porosus,* and Freshwater crocodile, *C. johnstoni*	Muir and Cunningham, 1990 Muir, pers. comm.
Fusarium sp.	Nile crocodile, *C. niloticus*	Foggin, 1992, pers. comm
F. solani	Snake eggs, *Elaphe guttata*	Kunert *et al,* 1993
Fusarium sp.	*Crocodylus* sp.	Youngprapakorn *et al*, 1994
Fusarium sp.	Turtle eggs, *C. caretta*	Gyuris, pers. comm.

Table 2: *Fusarium* infections in Reptiles.

Fungal pathogen	Species affected	Reference
Fusarium spp.	Birds, including egg embryos, pig	Ainsworth & Austwick, 1955
F. culmorum	Carp, *Cyprinus carpio*	Hörter, 1960
Fusarium sp.	Horse keratomycosis	Mitchell and Attleberger, 1973
F. solani	California Sea Lions, *Zalophus californianus* Gray seals, *Halichoerus grypus*	Montali *et al,* 1981
Fusarium spp.	Amphibians, birds, mammals, (review)	Austwick, 1984
F. oxysporum	Red Sea bream, *Pagrus major*	Hatai *et al,* 1986
F. solani	Pupfish, *Cyprinodon macularius* Triggerfish, *Melichthys vidua*	Ostland, 1987
F. solani	Baby Bonnethead Sharks, *Sphyrna tiburo*	Muhvich *et al*, 1989
F. solani	Baby Bonnethead Sharks	Smith *et al*, 1989

Table 3: *Fusarium* infections in Vertebrates other than Reptiles.

Fungal pathogen	Infection type and /or predisposing factor	Reference
F. solani	Oculomycosis	Jones, 1969, 1975
Fusarium spp.	Wide range of sites, review	Rebell, 1981
Fusarium spp.	Burns	Wheeler *et al*, 1981
Fusarium spp.	Eyes, skin, nails, subcutaneous and systemic, (review)	Austwick, 1984
Fusarium sp.	Mycotic keratitis, systemic after burns	Fetter and Klintworth, 1988
F. solani	Leukaemia	Venditti *et al*, 1988
Fusarium species (various), including *F. solani*	Invasive cutaneous and systemic infections (review), organ & marrow transplants, burns, carcinomas, trauma.	Rippon, 1988
Fusarium sp.	Peritonitis from dialysis	Chiaradia *et al,* 1990
Fusarium sp.	Toe trauma, leukemia	Nadler, 1990

Table 4: *Fusarium* infections in Humans.

Fungal pathogen	Species affected	Reference
Aspergillus sp., *Mucor* sp.	Freshwater and terrestrial turtles	Hunt, 1957
(a) *B. bassiana*, *Aspergillus* sp., *Geotrichum* sp., (b) *B. bassiana*, *P. fumoso-roseus*, (c) *B. bassiana*	(a) Galapagos Tortoise *T. elephantopus* (b) Aldabra tortoise *T. gigantea elephantina* (c) Turtles, *Terrapene carolina*	Georg *et al,* 1962
Various species (review)	Various species (review)	Reichenbach-Klinke and Elkan, 1965b
(a) *Cladosporium* sp. *(b)Basidiobolus ranarum*	(a) Anaconda, *Eunectes murinus* (b) Frogs, toads, lizards	Marcus, 1971, 1981
Cladosporium sp.	Tiger salamander	Migaki and Frye, 1975
Dermatophilus congolensis	Australian Bearded Dragon *Amphibolurus barbatus*	Montali *et al,* 1975
Geotrichum candidum	Captive carpet snake, *Morelia spilotes variegata*	McKenzie and Green, 1976
Aspergillus spp.	Green Sea turtle eggs, *C. mydas*	Solomon and Baird, 1979 in Wyneken *et al*, 1988
Sporotrichium sp., *Cladosporium* sp., *Paecilomyces* sp.	Green Sea turtles, *C. mydas*	Jacobson *et al,* 1979
Mucor sp.	Turtles, *Trionyx ferox*	Jacobson *et al,* 1980
Various	Snakes, various species	Jacobson, 1980
G. candidum	Giant Tortoise, *Geochelone elephantopus*, Turtles, *T. horsfieldi*	Ruiz *et al*, 1980
Paecilomyces spp., *Penicillium* spp., *F. solani*, *A. terreus*	Green turtles, *C. mydas*	Glazebrook, (1980) in Connole, (1990)

Table 5a: Fungal diseases reported in Reptiles.

Fungal pathogen	Species affected	Reference
Various (review)	Various (review)	Austwick and Keymer, 1981
Not identified	Gila monster, *Heloderma suspectum*	Hatkin, 1984
Various (review)	Various (review)	Migaki *et al,* 1984
P. lilacinus	Aldabra tortoise *T. gigantea elephantina*	Heard *et al,* 1986
Not identified	Eggshells of Leatherback Turtle *Dermochelys coriacea*	Solomon and Tippett, 1987
Possibly *Mucor* sp.	Loggerhead turtle eggs, *C. caretta*	Wyneken *et al*, 1988
Various species	Several reptilian species	Muir and Cunningham, 1990
Various species	Various, comprehensive review	Frye, 1991

Table 5b: Fungal diseases reported in Reptiles.

1.4.2 Fungal disease in Crocodilia

In an investigation of cutaneous lesions of an aged captive American crocodile (*C. acutus*), Jasmin and Baucom (1967) reported the presence of a bacterium *Erysipelothrix insidiosa* as well as *Mucor* sp., *Rhizopus* sp. and *Aspergillus* sp. Hyphae were demonstrated in the dermis from the tail. Lesions also were present along the jaw area extending into the buccal cavity around the base of the teeth. The fungi were considered to be secondary invaders (Jasmin and Baucom, 1967). Several six to eight week old caimans (*Caiman crocodilus*) from the same location also had necrotic foci under the scales of the abdomen and under the jaw and were found to be infected by the same bacterium. However, hyphal elements were not observed histologically (using standard haematoxylin and eosin stain). Some caiman hatchlings were also suffering from omphalitis. Treatments consisted of increasing the chlorination of the water and spraying the lesions with a 1% iodine solution. Penicillin doses were also incorporated into the animals' food.

Aspergillus fumigatus and *A. ustus* were isolated from the lungs of captive *A. mississippiensis* juveniles (two to six weeks of age) by Jasmin *et al* (1968). The disease manifested in the alligators as greyish nodular areas of necrosis in pneumonic

areas of the lungs, with hyphae evident in the tissue. Some slight desquamation of the scales of the abdomen and areas around the jaws and nostrils occurred and a few animals had necrotic areas between the tail, head and dorsal spines. Skin scrapings from these areas revealed the presence of fungal hyphae. *Rhizopus* sp., *Aspergillus* sp. and *Penicillium* sp. were isolated from the skin and scales but were not considered to be of significance (Jasmin *et al,* 1968). Omphalitis and bacterial presence in some tissues were also reported. Treatments which reduced mortality were chlorination of water, scrubbing the pens with 1:1500 copper sulphate solution, removal of visibly ill animals and provision of extra sunshine in the pens.

Zwart (1968) cited in a review by Frank (1975) reported mycotic infections in the lungs of a crocodile and also a caiman, however no further details of the causal pathogen or of the species of crocodilians affected were given in the review.

Candida albicans was identified as the agent responsible for pneumonia in unidentified species of crocodile and caiman (Zwart, 1968, cited in Jacobson, 1984, 1989). This report is assumed to relate to the same crocodilian mycosis as that reported by Zwart (1968) cited in Frank (1975).

Cephalosporium sp. was identified from the lungs of three 6 month old caimans (*Caiman sclerops*) as reported by Trevino (1972). Fatal diffuse granulomatous pneumonia and focal necrotizing hepatitis were found at necropsy of each animal. Numerous granulomas were observed in the lungs and liver. Other contributing factors to a debilitated state and lowered resistance to disease may have been nematode infections and poor adaptation to the captive environment (Trevino, 1972).

Keymer (1974) reported that pulmonary mycosis associated with *Penicillium lilacinum* was the probable cause of death of an alligator (*A. mississippiensis*) and a Nile crocodile (*C. niloticus*). Three species of fungi (*P. lilacinum, B. bassiana* and *Fusarium* sp.) were isolated from the lungs of a broad-fronted crocodile (*O. tetraspis*) which was also bacterially infected with *Pseudomonas aeruginosa* and was suffering from an infected bite wound on the leg.

Goodwin (1974) reported chronic respiratory and systemic mycoses in which *P. lilacinum* was isolated from five of six crocodiles and alligators. The extensive granulomata closely resembled tubercular lesions. In one Nile crocodile the fungus was accompanied by *Beauveria bassiana*, and in a Broad-fronted crocodile by

Metarrhizium anisopliae, which are both insect pathogens. Pathogenicity tests with *P. lilacinum* and *M. anisopliae* in lizards were confirmatory (Goodwin, 1974). Some of the cases reported by Goodwin (1974) may be the same as those reported by Keymer (1974).

Keymer (1976) reported that *P. lilacinum* was again diagnosed as the probable cause of pulmonary mycosis and death of an alligator (*A. mississippiensis*), a spectacled caiman and broad-fronted crocodile (*O. tetraspis*). The fungus was isolated from the lungs of the alligator and caiman but not the crocodile, and in all three cases the fungus was associated with an extensive and chronic type of pneumonitis. In the caiman a bacterium, *Aeromonas liquefaciens,* was also isolated from the lungs and liver, and was possibly the primary infection.

Silberman *et al* (1977) reported that *Mucor* sp. was isolated from lesions in the lungs and intestinal tract of four crocodiles from three species (*Crocodylus moreletii, C. acutus* and *C. niloticus*). All infected crocodiles died soon after showing initial clinical signs without responding to intensive antimicrobial therapy. The animals were housed in a common pool holding 32 animals from eight different crocodilian species, with a range of age groups from sub-adult to mature breeders. The stress of the crowded housing situation and the rancidity of the water in the pool which had not been cleaned for some time were considered to be contributory factors to infection (Silberman *et al,* 1977).

Austwick (1977) reported about seven cases of mycoses in the lungs of crocodiles at the London Zoo. Even though "aspergilloma" were produced, they were not caused by *Aspergillus* but by a series of other fungi, amongst them the pathogen *B. bassiana.*

Reichenbach-Klinke (1977) cited in a report by Boede and Velasco (1993a) frequently isolated *Microsporum nanum* and *Mucor micheli* from dermal lesions of crocodilians and other reptiles under captive conditions.

Goodwin (1978) and Jones (1978) both separately reported cases of respiratory mycosis which appear to be of the same animals at a zoo. Two *A. mississippiensis* died of chronic pneumonitis with *Paecilomyces farinosus* isolated from the first case and *Metarhizium anisopliae* from the second. Mycotic gastritis in the form of large chronic mucosal ulcers in a Nile crocodile (*C. niloticus*) appeared to have been caused by *Mucor circinelloides.* No significant bacteria were isolated from lesions and

histological examination of tissue confirmed the presence of widespread fungal infection in all three cases (Jones, 1978). The fungi isolated are normally insect pathogens however attempts to find the fungi in insects trapped near the exhibit failed (Jones, 1978) and environmental sampling failed to reveal other sources of those infections (Goodwin, 1978). The pathogenicity of the fungi was confirmed experimentally (Goodwin, 1978).

Kuttin *et al* (1978) reported two cases of mycoses in crocodiles kept at a German zoo. The first case was a twenty year old female Nile Crocodile, *Crocodylus niloticus* Laurenti 1978, which had stopped eating shortly after disinfection of its pool water by calcium hypochlorite. Lesions were observed in the mucous membranes of the oropharynx. The crocodile died after a further three months and a subsequent autopsy and mycological and histological examinations led to a diagnosis of *Trichosporon* sp. infection. The cause of death was considered to be the initial disinfection of the pool. Ingestion of the strong bleach caused mucous membrane ulcers and oedema, an extended period of starvation and also liver degeneration. The mycotic infection was considered to be secondary, facilitated by trauma caused by the calcium hypochlorite. The second case was a young male Brown Caiman (*Caiman crocodilus fuscus* Cope 1868) from the same pool as the first case crocodile. It was reluctant to feed and also showed lesions in the oropharyngeal mucous membranes. Biopsies and subsequent mycological and histological examinations resulted in the identification of both *Trichosporon* sp. and *Fusarium* sp. After sensitivity tests, the lesions were treated with chloramphenicol and an anti-fungal agent Amphotericin B, initially by topical application. Subsequently, intramuscular injections into the tail of the caiman using only the Amphotericin B were effected. The crocodile continued to decline and was euthanased. Autopsy revealed extensive haemorrhages in both lungs. Cause of death was considered to be haemorrhage in the lung brought about by physical trauma caused during handling of the crocodile while injecting the Amphotericin B. Calcium hypochlorite damage was seen as a contributing agent in causing the initial mycoses. A lack of sunlight, normally used by crocodiles for natural basking and disinfection, and lack of access to other artificial UV sources, was also considered to be a contributing factor.

Fromtling, Jensen *et al* (1979) reported the deaths of two captive alligators (*A. mississippiensis*) due to fatal pulmonary disease. *B. bassiana*, an entomopathogenic fungus, was isolated from pulmonary lesions in both animals. Virtually all bronchial and alveolar spaces were occluded with hyphae and the diagnosis of fungal pneumonia was made (Fromtling, Jensen *et al,* 1979). Granulomas with hyphae-filled necrotic centres were also present in the liver and spleen however the lungs were considered to be the primary focus of infection. Predisposition to infection was thought to be due to an extended hibernation period by the alligators in an unusually severe winter and a heating failure in the exhibit. It was suggested that the animals ingested or inhaled the spores before hibernation and harboured the chronic infection throughout the hibernation period. Two remaining alligators were given a series of multi-vitamin injections.

The death of a third captive *A. mississippiensis* infected with *B. bassiana* was recorded by Fromtling, Kosanke *et al* (1979). Previous mortality in the same group of alligators caused by the same pathogen was reported by Fromtling, Jensen *et al* (1979). This third animal had also been subject to an extended cold period and had become lethargic and refused to eat. At necropsy an estimated 60% of the pulmonary tissue was damaged by fungal growth, with extensive colonies forming 'fungal mats' within the parenchyma. The colonies had sporulated *in vivo*, which may have permitted dissemination of spores into the air when the infected animal exhaled (Fromtling, Kosanke *et al,* 1979). Deep invasion of hyphae into the visceral and parietal pleurae had also occurred although there was no evidence of fungal involvement in the heart or any other organ outside the thoracic cavity. Utz *et al* (1977) cited in Fromtling, Kosanke *et al* (1979) had previously recorded the formation of "pulmonary fungus balls" by *B. bassiana* in crocodiles.

Fusarium sp. was found in cutaneous infections in a caiman (Jacobson, 1980, cited in Marcus, 1981). No other details were given in Marcus (1981).

B. bassiana was the fungal pathogen isolated from alligators which succumbed in one "major die-off" at a zoo (Jacobson, 1981).The alligators had been kept at 15°C and pneumonia and dermatitis appeared to be the most common signs of the fungal infections. Suggested treatments for fungal infections were Nolvasan (cyclohexadine),

Betadine (iodine based), malachite green or Tenactin, a product used for human superficial mycoses (Jacobson, 1981).

Austwick and Keymer (1981) carried out a major review of reptilian diseases which included many references to crocodilians. Some of the cases cited by Austwick and Keymer (1981) have been detailed separately in this literature survey. Some reports also seemed to be of the same cases reported individually by more than one author (*eg.* Goodwin, 1974, and Keymer, 1974; Goodwin, 1978 and Jones, 1978). Cases surveyed by Austwick and Keymer (1981) which have not been dealt with elsewhere in the literature survey include:-

- *A. fumigatus* isolated from the lung, liver, spleen, myocardium and intestine of an *Alligator* sp. (Pallaske, 1957, cited in Austwick and Keymer, 1981);
- *Penicillium* sp. in the lung of an *A. mississippiensis* (Williamson *et al*, 1963, cited in Austwick and Keymer, 1981);
- unidentified fungus forming a projecting nodule, with hyphae present, on the head of an *A. mississippiensis* (Plusinski, 1966, cited in Austwick and Keymer, 1981);
- unidentified fungal pathogen from the skin of a *Crocodylus* sp. (Pooley, 1971, cited in Austwick and Keymer, 1981);
- the isolation of *Metarhizium anisopliae* from the lungs of a Nile crocodile (Keymer, 1974, cited in Austwick and Keymer, 1981);
- secondary growth of *A. niger* on airway mycelial plaques of *P. farinosus* in an American alligator (Jones, 1978, cited in Austwick and Keymer, 1981).

Migaki *et al* (1984) comprehensively reviewed fungal diseases in reptiles. In one report a *Trichophyton* sp. was isolated from granulomatous lesions of the foot pads of a juvenile alligator and hyphae were demonstrated histologically in the tissue sections.

Austwick (1984) reported *Fusarium* sp. infections in alligators and crocodiles. The lungs were affected in one *A. mississippiensis* and one *O. tetraspis*, and the skin was the site of infection in one *C. niloticus*. No other details were given, however, it was noted by Austwick (1984) that "although no experimental inoculations have been reported, it seems likely that certain fusaria *eg. F. solani*, will eventually prove to be primary pathogens of reptiles as well".

Foreyt *et al* (1985) captured a single juvenile female *A mississippiensis* covered with a fungus-like material. The animal died within 24 hours from an unrelated accident and

skin biopsies were taken from its neck and legs. A *Trichoderma* sp. was identified from culture and corresponding hyphae were seen histologically. Protozoans were also seen in the cutaneous layer of the keratin. The heavy but superficial growth of *Trichoderma* sp. was not considered pathogenic, however the generalised growth of the fungus on the juvenile alligator may have reflected an opportunistic infection secondary to malnutrition or other illness (Foreyt *et al,* 1985).

Fatal pulmonary infection in a captive *A. mississippiensis* was reported by Frelier *et al* (1985). No clinical signs were present before death and at necropsy the animal appeared to be in excellent nutritional condition. Multiple white necrotic foci were scattered throughout the lung parenchyma and many dilated bronchi were lined with plaques of fungal hyphae. Histologically, fungal elements were demonstrated in the lung, with the presence of single or chains of conidia seen within the lumen of the bronchioles. The isolate was identified as *Fusarium moniliforme*. As the alligator appeared healthy before death, the infection was thought to have been due to exposure to contaminated material (Frelier *et al*, 1985). Stress may also have been a contributing factor as the alligator's death coincided with an artificial cold period and semi-hibernation, resulting in reduced food consumption and activity. Other reports of mycotic infections in crocodilians by Kuttin *et al* (1978) and Jacobson (1980) were both cited in Frelier *et al* (1985). Those reports stated that the integument and lungs were the two organ systems most commonly involved, with *Trichophyton*, *Aspergillus*, *Mucor*, *Rhizopus*, *Trichosporon* and *Fusarium* spp. isolated from cutaneous lesions, and *Candida albicans*, *Acremonium* (= *Cephalosporium*) and *Mucor* spp. isolated from pulmonary lesions. The full details of the report by Kuttin *et al* (1978) have been described elsewhere in this literature review.

Foggin (1987) reported the results of post-mortem examinations of 6 Nile crocodiles, *C. niloticus*, from crocodile farms in Zimbabwe. Fungal granulomatous hepatitis in one case, and fungal granulomatous pneumonitis in two cases were considered to be primary causes of death. Other cases involving these two infections had been diagnosed histopathologically, but they appeared to be secondary infections and were not considered important. One case of fungal stomatitis and two cases of fungal dermatitis were incidental to the main cause of death. Fungal dermatitis was a common condition, in which the dorsal skin appeared dry with a fine white coating

but in the mouth a more proliferative reaction was present. On histopathological examination fungal spores and hyphae were demonstrated in the superficial epidermis. At the time of that report, the fungal species had not been identified and the infection usually disappeared spontaneously. Foggin (1987) reported that treatment for fungal dermatitis was the use of potassium permanganate in the pond water at 10 ppm.

Webb, Whitehead and Manolis (1987) reported that "a fungal infection causing severe skin lesions and increased mortality of *C. johnstoni* hatchlings occurred in two consecutive years" and was one of the major problems encountered at one crocodile farm in the Northern Territory, Australia. The pathogen (not named) had proved difficult to control within low-cost low-maintenance pens and at the time of their report those pens were being overhauled to remove earthern banks and to increase water flow. The disease had not appeared at other farms even after transfer of animals from the infected farm when it was thought the disease was under control. Except for pen overhaul no other methods of pathogen control were detailed.

Onions (1987) reported that the major problem for a crocodile farm in the Northern Territory, Australia was a fungal infection of hatchlings, with types of pens previously used abandoned. No details of the pathogen or of its manifestation were detailed. This report may refer to the same problems and farm as that discussed by Webb, Whitehead and Manolis (1987). Onions (1987) also stated that unexplained mortality in crocodiles, particularly hatchlings, was another important problem for all crocodile farms in Australia.

Maslen *et al* (1988) described the findings of an investigation into the sudden death of two five month old hatchling *C. porosus* bred and raised in captivity. Tissue from the first hatchling was autolysed. At autopsy, multiple granuloma-like lesions were seen in the liver, left lung and spleen from the second hatchling and hyphae were viewed histologically in sections of liver and spleen. *P. lilacinus* was isolated from liver lesions. A culture from a subcutaneous abscess on the hatchling's left thigh was overgrown with bacteria however it was thought that the fungus was present in the abscess (Maslen *et al*, 1988). The small size of the hatchling indicated that it may have been a weakling and therefore more susceptible to disease. It was suggested that the opportunistic fungal pathogen gained entry from the environment through the damaged skin (possibly a bite wound caused by another hatchling) or alternatively via

live goldfish which had been introduced to the hatchlings' diet shortly prior to death (Maslen *et al*, 1988).

In a study of diseases of farmed crocodiles, (*C. porosus and C. johnstoni*) Ladds and Donovan (1989) observed that mycotic infections which manifested as pale fungal granulomas in the liver and lung seemed to occur as isolated lesions and were not considered as a major cause of illness. The fungal pathogens involved were not named in that report. Branched filamentous organisms, similar to *Dermatophilus congolensis* (a pathogenic Actinomycete) had been observed microscopically in ulcerative skin lesions in young crocodiles up to 2m in size. However, the pathogen had not been cultured at the time of the report.

Jacobson (1989) again reviewed diseases of crocodiles, however all except one of the cases cited have been dealt with individually in this literature review. The additional report was of a skin disease in farm reared American alligators (Jacobson and Cardeilhac, unpublished findings, cited in Jacobson, 1989). The disease manifested as circular raised lesions which histologically consisted of focal areas of epidermal necrosis, the surfaces of which were often covered with spore-like structures. Although the causative agent was not isolated, branching filamentous structures compatible with those of *D. congolensis* were observed by light microscopy.

Freeman (pers. comm.) advised that fungal disease was a problem in *C. johnstoni* hatchlings and *C. porosus* yearlings at a North Queensland crocodile farm. Up until late 1990 there had been no losses but the disease had not been totally eradicated, nor the causal agent identified. Up to 60% of *C. porosus* yearling stock had been affected to some degree over a period with approximately 15% still seriously affected. Attempted treatment was by spraying the crocodiles with Betadine, using chlorinated water in the ponds, and cleaning the pens using Parvocide.

Muir and Cunningham (1990) reported mycotic infections in farmed crocodiles, both *C. porosus* and *C. johnstoni*, in the Northern Territory, Australia, as well as mycoses caused by a variety of fungi in a range of other mostly captive reptiles. All crocodiles had skin lesions of varying severity. From information provided (Muir, pers. comm.), a summary of the fungal species isolated from *Crocodylus* sp. is listed in Table 6. Most animals were hatchlings less than one year old unless otherwise noted. *F. solani*

was also isolated from a File Snake and a Pig-nosed Turtle. *P. lilacinus* was also isolated from a Long-necked Turtle, a Galapagos Tortoise, and an American Turtle.

Crocodile species	Fungal species	No.	Site
C. porosus	*F. solani*	6	fore-feet and skin
	F. oxysporum	5	skin
	Mucor circinelloides	3	skin
	Myceliophthora sp.	3	skin
	Aspergillus oryzae v. effusus	1	skin
	Curvularia eragrostidis	1	skin
	Phoma sp.	1	skin, yearling
	Paecilomyces variotii	1	skin, yearling
	C. clavata	1	skin, yearling
	A. versicolor	1	skin
	Nigrospora sphaerica	1	lung, liver, kidney, skin
	Chrysosporium lucknowense	1	skin, neck, tail, foot
	P. lilacinus	1	liver, two year old
	P. lilacinus	1	skin
	C. lunata	1	hind-feet, tail, belly
	F. equiseti	1	skin
	A. flavus	1	skin
	Beauveria bassiana	1	skin
	Penicillium oxalicum	1	skin
C. johnstoni	*Arthrographis* sp.	1	skin
	A. niger	4	liver, skin
	F. equiseti	1	skin
	F. semitectum	1	skin
	C. lunata	1	feet, snout, eyes, belly

Table 6: Summary of fungi isolated from farmed *Crocodylus* sp. (after Muir and Cunningham, 1990, and Muir, pers. comm.) (No. = Number of isolates).

Schumacher & Cardeilhac (1990) described the isolation of four species of fungi from the egg membranes and chorioallantois of eggs from *A mississippiensis*. The eggs were collected from two alligator farms (Florida) and from free-ranging wild alligators (Louisiana). *Fusarium oxysporum* was isolated from 27 eggs and additional fungal species *Penicillium fellucanum, Paecilomyces aviotti* and *Aspergillus niger* were isolated from two of those eggs.

In a detailed study of diseases of juvenile crocodiles (*C. porosus* and *C. novaeguineae*) in Papua New Guinea, Ladds and Sims (1990) reported that the three

major infectious diseases were coccidiosis, bacterial septicaemia and metazoan parasitism. A range of other lesions and infective agents were recorded but no systemic fungal diseases were reported. *Dermatophilus*-like branching filaments were observed in some cutaneous lesions which were assumed to be the site of a traumatic injury. Adaptation failure and subsequent starvation were also common and stress was suggested as playing a major role in the pathogenesis of disease (Ladds and Sims, 1990). The juvenile crocodiles had been captured in the wild and then held on a commercial crocodile farm.

In a review of the most important diseases in Crocodilia which may affect human health through consumption of imported crocodile meat (*A. mississippiensis* and *C. niloticus*) in Belgium, Debyser *et al* (1991) reported that *Cephalosporium* sp. had been isolated from crocodile muscle lesions.

Onions (pers. comm.) advised that fungal infections affected small hatchlings (500-600 mm length, aged about twelve 12 months) after relocation over long distance using air transport. The fungus was first noticed around the hatchlings' eyes and snouts. Animals lost condition and usually died. Up until late 1991 losses in the first three months after each group's relocation were 2-3%.

In a news release by Antec International Ltd, (1991) it was reported that the use of Virkon S® (refer Appendix 1) had been tested in sensitivity trials at a Zimbabwean Veterinary Research Laboratory. Fungal infections causing superficial dermatitis, chiefly affecting one and two year old farmed crocodiles in Zimbabwe were threatening producers' profits. *Fusarium* sp. was reported as one of the causal agents of the infections which seriously affected hide quality. Results of testing showed that a solution of Virkon S® at 1:300 would successfully combat the problem. Farmers were reported to be using Virkon S® to clean pens, add to pond water, and spray over hatchlings.

Huchzermeyer (1991) reported a systemic fungal infection in Nile Crocodiles, *C. niloticus*, subjected to prolonged periods of cold. The pathogen was not specified in the report. However, the infection was characterised by granular lesions in the lungs and liver, and small white crater-like lesions on the pleura and peritoneum. The lung granulomas were associated with severe inflammatory reaction. Mildly affected animals had few visible symptoms. However, the course of the disease appeared to be

chronic. Growth rates of the infected animals were often unaffected until liver and lung damage was severe enough to cause mortality. Although only a few crocodiles developed terminal lesions, the incidence of infection may have been fairly high. No treatments of infected crocodiles were attempted and all cases were diagnosed from post-mortem examination. It was postulated by Huchzermeyer (1991) that prolonged exposure to cold effectively suppressed the crocodiles' immune systems thus allowing the fungal pathogen to become established. When the ambient temperature returned to normal, the pathogen was already well established in the crocodile host and the crocodile's re-activated defence system was unable to control the infection.

Buenviaje (1991) and Buenviaje *et al* (1994) reported the results of a major survey of disease-husbandry associations in farmed crocodiles in Queensland and the Northern Territory. Most of the animals examined by post-mortem, biopsy or retrospective records were juveniles, both *C. porosus* and *C. johnstoni*. Superficial mycoses were recorded from animals from two of four Northern Territory farms and two of three Queensland farms. Deep mycoses were recorded at two of three Queensland farms and mycotic gingivitis was recorded at one. The higher latitude of the farms which had deep mycoses was considered relevant as most cases occurred during the winter months. *F. solani* was repeatedly found as the causal agent of deep mycoses of crocodiles from one farm in Queensland (Hibberd, pers. comm. cited in Buenviaje, 1991; Hibberd and Harrower, 1991, cited in Buenviaje *et al,* 1994). Fungi isolated from superficial lesions on the head, belly, tail and between the scutes of crocodiles in the Northern Territory were identified as *A. niger*, *Penicillium oxalicum* and *Curvularia lunata varaeria*, however no particular fungus was incriminated as the primary pathogen. No details were given on the identification of the fungal pathogens causing deep or superficial mycoses at the other farms.

Fungal dermatitis was reported by Foggin (1992) as a problem in slaughter stock as well as other age groups of *C. niloticus.* Hide quality was affected by lesions between the scales and excoriation of the epidermis in the skin folds. In severe cases mortality occurred when the nares became blocked by exudate. *Fusarium* sp. and other mycelia-producing fungi were isolated in culture. Contributing factors were believed to be lapses in standard of hygiene, the type of animal housing, and the nutritional status of the animals.

Douglas and Douglas (1992) acknowledged that fungal disease affecting hatchling and yearling crocodiles was a problem for some Australian crocodile farmers and considered that it was the major disease which affected those crocodilian age groups. At the time of that report however, fungal disease had not been identified at their facility in Western Australia (Douglas and Douglas, 1992; Douglas, pers. comm.).

Ratanakorn (1993) reported that fungal disease caused by *Fusarium* sp. had been common in Thai crocodile farms over the preceding three years. However, the mycoses were considered to be secondary infections with most cases diagnosed from post mortem material. Other health problems were sudden death and anorexia in hatchlings, and undiagnosed embryonic mortality.

Superficial and deep mycoses were two of the three main causes of hatchling disease or mortality in crocodile farms in the Northern Territory, with G-ve bacterial hepatitis/septicaemia as the third (Melville, 1993). The fungal diseases were considered to be opportunistic infections and tended to be more severe in the farms at greater latitudes. Most mortalities from all diseases occurred in the cooler months when inadequate or no heating was provided. Superficial mycoses with discrete lesions and loss of digits were reported. Deep mycoses were seen as lesions in the intestine, lung and liver. A *Fusarium* sp. was identified as a common causal agent.

In late 1993, McInerney (pers. comm.) commenced investigations into possible water additives for use as treatment of intermittent superficial fungal infections on farmed *C. porosus* hatchlings in the Northern Territory. Formalin solutions had been used on some farms but severe side effects had resulted (McInerney, pers. comm., O'Brien, pers. comm.). Chlorinated water was used routinely with little effect. Animals presented by farmers to veterinarians for post-mortem were considered only a small fraction of those which may have been affected on the farms as only animals with 'unusual' symptoms were submitted.

Boede and Velasco (1993a) found high mortality in seven month old hatchlings of *C. crocodilus* on a large Venezuelan farm. Bacteriological and mycological studies were carried out. No fungal or bacterial treatments were reported. Symptoms described were white cutaneous lesions; erosive lesions on the lower jaw, dorsal and ventral body surfaces and tail; digit loss; eye inflammation with partial or total blindness; emaciation, anorexia and death. Bacteria identified were *Escherichia coli*

from the tongue, stomach, small intestine, trachea, and lungs, and *Proteus vulgaris* from the heart and liver. It was considered that the bacterial infections contributed to the high mortality. Three species of fungi were identified, *Microsporum nanum* isolated from the digits and jaw, *Mucor micheli* from the digits, and *Trichophyton simii* from the epidermis.

Boede and Velasco (1993b) reported seventy necropsies performed on two to four month old hatchlings from seven Venezuelan caiman farms between 1990 and 1993. A wide range of symptoms was observed. Bacteriological investigations resulted in an extensive list of bacteria recorded. A wide range of invertebrate parasites was also identified in the intestines and lungs. The only mycosis reported was an *Aspergillus* sp. infection of the skin. Fungal infection rates and severity were not reported. Because of the range of symptoms and results they were unable to give definitive diagnoses as to the primary causes of mortality. However gastric, intestinal and pancreatic atrophies were diagnosed, these resulting from poor absorption of nutrients related to poor body condition (runt syndrome). Gastro-intestinal problems then allowed secondary bacterial infections. The authors considered that the range of conditions observed was due to inadequate farm husbandry, chronic stress in the animals, unbalanced diets and excessive handling of the hatchlings.

In their colour atlas of crocodile diseases and abnormalities, Youngprapakorn *et al* (1994) reported mycoses in farmed crocodiles at the world's largest commercial crocodile farm in Thailand. Samutprakarn Crocodile Farm and Zoo holds over forty thousand live crocodiles. These include the endemic freshwater species *Crocodylus siamensis* Schneider 1801, *C. porosus*, the estuarine crocodile which is widespread throughout southeast Asia and the western Pacific as well as Australia, and hybrids of these species. Also farmed there is the endemic freshwater False Gharial *Tomistoma schlegelii* S. Müller 1838, and a range of other crocodilian species from other parts of the world. The authors reported three types of fungal disease. The first was a rare pulmonary mycosis with whitish yellow fungal granulomas covered with thick fibrous capsules throughout the pulmonary tissue. The aetiological agent was not reported, nor was the age of the animals affected nor the species. The second disease was a dermatomycosis with the causal agent identified as *Fusarium* sp. Lesions were present around the hatchlings' eyelids, external nares, forelimbs, lips and teeth. Species of

crocodile native to Thailand apparently survived the infection but were left with physical defects, however mortality was high in exotic species. Although not recorded in the text, one of the three hatchlings pictured with dermatomycosis is *C. siamensis*, the second is *C. porosus*, with insufficient photographic detail to identify the third (Lever, pers. comm.). The third disease reported was a subcutaneous mycosis severely affecting the ventral skin of the crocodiles. The animal illustrated in the atlas is a hatchling. However, the species is not identified. Superficial scales were swollen and loosened in the inguinal area and on the ventral surface of the hind limbs, with evidence of hyperaemia and oedema in the dermal and subcutaneous tissue. The aetiological agent was not identified. The atlas uses colour photographs of affected animals and post-mortem dissected tissues, and colour photomicrographs of stained histological sections. The authors stated that debilitating conditions such as gastric obstructions may predispose crocodiles to mycotic infections. Although not specifically referring to mycoses, the authors also stated that disease and deformities create great losses to crocodile farming, and that treatments after the onset of disease are almost always futile and ineffective. They considered that prophylaxis, and understanding disease aetiology would lead to successful crocodile farming with low morbidity and mortality. Fungi were not cultured from the post-mortem material however hyphae were seen in histological slides (Youngprapakorn, pers. comm).

C. porosus hatchlings used in temperature and feeding trials in a North Queensland research facility were found to have fungal infections (Mayer, 1994). The hatchlings were sourced from a North Queensland crocodile farm and on arrival were from age four to six weeks. A few from one tank (all from the same clutch) had a "skin disease manifested by patches of cream-white layers of skin that could be peeled off". The problem was identified as a 'ringworm', *Trichophyton mentagrophytes*. In the following ten days, a second fungal infection appeared in the same tank and spread rapidly to animals in a separate tank in the same room, infecting nearly every crocodile. The second fungus manifested as "numerous patches of grey-lilac furry growth on mainly the backs and heads of the animals". The aetiological agent of this second infection was identified as *P. lilacinus*. A series of treatments was applied to infected animals. These included adding formalin to the water in the tanks (5 mls per tank); swabbing fungal affected areas of the hatchlings with Betadine® and leaving

them out of the water to dry; and bathing the animals in salty water. Treatments continued over a four week period with an increasing concentration of formalin added to the water in the tanks (10 mls per tank, increasing to 20 mls per tank), and animals both swabbed and dipped in Betadine® and allowed to dry off. The researchers reported that "the initial concentration of formalin was too low, that the salt solution had no effect, and that the Betadine® solution had limited impact". The outbreaks were, however, brought under control with no obvious white fungal areas present by the time of cessation of treatments. Eight animals from the tanks which held infected hatchlings died during the treatment period. One other animal with a persistent distended stomach was euthanased and was found to have an unabsorbed yolk sac. One month later another particularly infectious outbreak of ringworm occurred in another research room. Twelve hatchlings died from this ringworm infection over the ensuing three weeks. The mode of transfer of the infection was a matter of speculation by the researchers as the two separately infected groups of animals were not in physical contact, nor were they from the same clutch. The second group was held in tanks with the lowest water temperature and it was surmised that the stress of this low temperature contributed to the fungal outbreak.

Langelet (pers. comm.) advised that fungal disease was suspected as one of the factors affecting below average hatchability in 1994 at a crocodile farm in Papua New Guinea. *C. porosus* eggs were sent to the National Veterinary Laboratory where *Trichophyton* and *Aspergillus* species were isolated from the inner and outer shell. Mortality among 0-6 month old hatchlings was less than 3% and was not considered a problem by the farm management (Langelet, pers. comm.). Other husbandry factors which contributed to the low hatchability were being addressed to prevent their recurrence and methods of disinfecting the eggs were being investigated. The fungi isolated from the eggs and nesting material were ubiquitous in the natural environment and veterinary advice was that fungal infection in the crocodile eggs was not a major problem. In early 1996 Langelet (pers. comm.) advised that *Mucor* sp. had been recently isolated also.

Huchzermeyer and Agnagna (1994), in a bacterial and fungal examination of faecal samples from 21 mature African Dwarf Crocodiles (*Osteolaemus tetraspis*), found an average of 3 fungal species per sample. These are listed in Table 7. All the

animals had been captured from the wild within the previous 30 days for sale and slaughter at village markets and were severely stressed possibly due to prolonged water deprivation and exhaustion.

Genus	Species	No. isolates
Aspergillus	*clavatus, flavus, niger*, sp.	8
Penicillium	sp.	7
Cryptococcus	*lipolytica, luteolus*	4
Beauveria	sp.	3
Candida	*guilliermondii, krusei*	3
Chrysosporium	sp.	3
Trichosporon	*beigellii, capitum*	3
Paecilomyces	sp.	2
Acremonium	sp.	1
Arthrinium	sp.	1
Curvularia	sp.	1
Phoma	sp.	1

Table 7: Fungi isolated from intestinal contents of *Osteolaemus tetraspis* (Huchzermeyer and Agnagna, 1994).

An outbreak of fungal infection in *C. porosus* hatchlings at a crocodile farm in Indonesia was recorded by Cannucciari (pers. comm.). The effect observed on the hatchlings was similar to that often reported on fish affected by *Saprolegnia* sp. (Reichenbach-Klinke and Elkan, 1965a). White cottony growths were located mostly around the mouth, eyes, and feet of hatchlings, or wherever else lesions or scars were present. The affected hatchlings were treated by dipping them for fifteen to thirty minutes in a potassium permanganate solution, the concentration of which varied from 0.5 to 1.0%. The outbreak was not severe as mortality was limited to two hatchlings, while four which were only mildly affected recovered after treatment. Several weeks later all the hatchlings were transferred to new pens under different conditions and no further outbreaks were reported up to April 1995. Laboratory analysis confirmed the fungus as *Saprolegnia* sp.

Foggin (pers. comm.), using specimens from *C. niloticus* farms in Zimbabwe and recorded after previously published reports (Foggin, 1987, 1992) and up to mid 1995,

continued to see at post-mortem, fungal infections with three separate syndromes. Neonatal skin infections, which grew "rapidly as a land-like (*sic*) coating on the skin, especially on the head, rather like *Saprolegnia* sp. on the scales of fish", caused high mortality, and were believed to be associated with contaminated nesting material. Malachite green (1-2 g per 2000 L) was used as treatment. Fungal dermatitis associated with inflammation of the dermis and epidermis with fungal hyphae penetrating those structures was also encountered. It was often secondary to pox or was associated with poorly ventilated and high humidity housing. Poor water quality was also implicated. Prevention and treatment was by improved ventilation, access to sunlight and allowing the crocodiles to bask. Systemic fungal infection, usually in the lungs and sometimes in the liver of hatchlings was also seen. Occurrence was sporadic on Zimbabwean farms but an outbreak was recorded on a Ugandan farm in 1994 (Foggin, pers. comm.). The main feature of the systemic infections was necrotic granulomatous lesions with fungal hyphae demonstrated in the lesions by histological staining techniques. No treatment attempts were reported for systemic infections. *Fusarium* species were the most commonly isolated fungi from all syndromes (Foggin, pers. comm.).

In a study of diseases of juvenile crocodiles (*C. porosus* and *C. novaeguineae*) in Irian Jaya, Ladds *et al* (1995) reported only one case of fungal pneumonia from thirty eight post-mortems. The identification of the fungal pathogen was not reported. The hatchlings with an age range of from two months to two years had been collected from the wild and grown in captivity.

The majority of the cases of fungal disease in crocodilians detailed in section 1.4.2 have been from captive animals in zoos or similar, or from farm bred stock. Some exceptions to this were the reports by Foreyt *et al* (1985) who sampled a captured wild *A. mississippiensis,* Schumacher and Cardeilhac (1990) who sampled both wild and captive laid *A. mississippiensis* eggs, and Huchzermeyer and Agnagna (1994) who sampled recently captured wild *O. tetraspis.* Ladds and Sims (1990) and Ladds *et al* (1995) carried out post-mortems on juvenile *C. porosus* and *C. novaeguineae* which had been captured from the wild and held on crocodile farms.

Treatments where attempted were frequently ineffective, and the majority of reports were from moribund or dead animals.

2. MATERIALS AND METHODS

2.1 Preliminary Laboratory Observations

In order to make daily observations on the progress of the disease symptoms, four yearling crocodiles were brought to the CQU campus during a three month period from 7 June to 17 Sept 1990. All animals had ceased to feed of their own accord, were very lethargic, and had similar symptoms to others which had recently died (Lever, pers. comm.). On arrival each animal was weighed and the snout to vent length and overall body length recorded. Each yearling was identifiable by its 'scuted' group number. This was the method used at the farm at that time to identify animals from a particular group. (Lever, pers. comm.) These groups were made up of progeny from different breeding female crocodiles leased by a single investor and could, therefore, be a mix of unrelated hatchlings from different clutches. 'Scuting' is a standard crocodile farming practice usually carried out when hatchlings are one week old, and involves cutting off some of the vertically raised scales along the dorsal body surface. These scales form a Y pattern with a single row along the tail, and two divergent rows from a junction midway along the tail extending anteriorly. When viewed dorsally and starting from the Y-junction and counting anteriorly, scutes removed from the right side were given numbers from zero to nine, and those removed from the left side were numbered from zero in multiples of ten. Thus a hatchling from group 6 would have only the seventh scute on the right removed, group 10 would have only the second scute on the left removed, and group 12 would have the second on the left as well as the third on the right removed. This method of identification did not differentiate between years of hatching. When carried out using sharp sterile implements (*eg.* scalpel) and with the scuted area swabbed with antiseptic before and after cutting, the wound healed quickly without any regrowth of the scutes. Hatchlings and yearlings could often be identified by observing their tail scutes without having to pick up or otherwise disturb them.

Initially three sickly yearlings were placed under observation at the same time and in the same tank, one each from groups 6, 10, and 11. After the early subsequent deaths of yearlings 6.1 and 10.1, a second yearling from group 11 was obtained (11.2 together with 11.1) for further observations.

One culture room in the CQU Biology Department's animal house was made available, initially as sole usage but later due to space restrictions, as shared facilities. Due to problems associated with this sharing, the animals were shifted to a new observation tank in another smaller unused room. Both rooms had a reverse cycle air-conditioner for ambient temperature control, with fluorescent lighting set to a main building timer giving a regime of eleven hours dark and thirteen hours light (6 am to 7 pm). Both rooms were without windows and were accessed via a single door. The rooms and observation tanks were set up and stabilised prior to use. Room air temperature was recorded daily using a max/min thermometer. Tank water temperature was recorded similarly with a max/min thermometer placed in the water at the bottom of the tank. Humidity levels were recorded daily using a wet and dry bulb thermometer. High humidity levels were maintained by the volume of water in the observation tank, with additional moisture provided by hosing the floor daily.

The first observation tank was a standard glass aquarium, 905 mm long, 385 mm wide, and 385 mm high. This was later superseded by a second observation tank which was purpose-built from glass but with dimensions of 1350 mm long, 500 mm wide, and 400 mm high. Both tanks were surrounded on three sides by a sheet of black plastic attached to the exterior, thus enabling worker entry to and exit from the room without unduly disturbing the animals. A double fluorescent light fitted with two Sylvania 36W Grolux plant lights was suspended over the tanks to give additional UV light. An Osram Siccatherm 250W infra-red lamp was also positioned externally at the uncovered end of the tanks at water level height, to allow basking by the animals. Initially both the ultra-violet and infra-red lamps were connected to a timer, which turned them on from 8 am to 6 pm simulating normal daytime conditions. The infra-red lamp was later left on permanently. A standard aquarium heater was installed underwater in the water inlet end of the tank (opposite end to the infra-red lamp) to help maintain a stable water temperature. The heater was placed inside a protective solid PVC sheath which had been drilled repeatedly to enable free water flow but which prevented damage to the heater by the animals. The wiring was also protected. A sloping ramp was fitted inside the tank to allow basking by the crocodiles, either in or out of the water. The animals also used the ramp for hiding under whilst fully submerged or when partially immersed with head raised. Three large smooth river

rocks were positioned inside each tank to enable easy access up the ramp by the crocodiles, and to allow basking both out of the water or partially submerged. Before use, these rocks were scrubbed with detergent and disinfectant to remove obvious organic material. For further decontamination they were then soaked for 4.5 hours in a 25% bleach solution (Glochlor, refer Appendix 1) followed by extensive rinsing with running tap water. The tanks were filled to a depth of 150 mm with warm water (approx 32°C). After first moving the animals to a separate covered tub, the first tank was emptied daily using a siphon hose, cleaned, then refilled with warm water. The second tank was fitted with a system of pipes at one end which allowed draining, flushing and refilling of the tank without the necessity of first removing the animals. The indoor aquarium with hatchling *C. porosus* is pictured in Plate 3a. Plate 3b depicts hatchlings housed in indoor heated pens at the farm.

For crocodile body length and snout to vent length measurement, a simple frame was constructed from a sheet of acrylic 150 mm wide by 700 mm long. This sheet was bent 100 mm from one end at an angle of 90 degrees to give an L-shape. A 600 mm length of fibreglass tape measure was then permanently attached to the centre line of the horizontal portion with the zero mark coinciding with the vertical. Measurement was effected by placing the crocodile on the measuring board with its head abutting the vertical section and the body and tail over the tape measure. This could be done with the crocodile's ventral or dorsal surface uppermost. Acrylic and fibreglass materials were used to allow easy disinfection of the board with 25% bleach between measurements of different crocodiles. Each crocodile was measured on arrival and at death.

Body weight for each crocodile was recorded on arrival, then daily prior to feeding. The animals were removed from the observation tank and weighed in a wire mesh basket on a Mettler TE6/J balance (to 6 kg ±2 g). Their weights were also recorded after their deaths.

Food was fresh lean beef steak and kidney, or calf liver, diced into pieces of up to 1 cm^3. The food was weighed before being initially offered to the crocodiles by placing on the dry sections of the basking platform or on one of the rocks. Tying light cotton thread to the meat and then moving the meat about near the animals was also trialed,

Plate 3a: Juvenile *C. porosus* maintained in indoor aquarium.

Plate 3b: *C. porosus* hatchlings in heated indoor pens.

as was dropping the meat near the snout of the animal. Force-feeding was later used as a last resort to prevent total anorexia. This was effected in various ways.

By holding the crocodile with its head raised and jaws open, a pre-weighed piece of meat could be dropped into its mouth. The crocodile usually only swallowed if the meat fell to the rear of its throat at or near the palatal valve. A second method involved skewering the meat onto a blunt wooden meat skewer, and then using the skewer to push the meat beyond the palatal valve into the crocodile's throat. Massaging its neck assisted or promoted swallowing. Uneaten or regurgitated food was collected from the tank and the weight of food consumed by each animal was recorded. To enable nutrient intake in the third observation crocodile, force feeding using Heinz Beef and Vegetable baby food was trialed. A modified 5 ml syringe with silicone tubing attached was used to feed the animal, following the drenching gun procedure as outlined in section 2.5. Food supplements of iodised salt and calcium di-phosphate (refer Appendix 2) was added to the meat fed to the fourth crocodile received for observation.

Evidence of defecation was checked daily as an indication of food processing and assumed nutrient absorption by the crocodiles. Similarly, regurgitated food was also checked.

Treatment of the fungal infections in the third and fourth crocodile was attempted by using Nitrofurazone (refer Appendix 1). In the early stages of the trial, the powder was weighed (to give a final observation tank dilution of 10 mg/l), mixed with five millilitres of ethanol to dissolve, then diluted to approximately 100 mls with water. Later in the treatment trial the Nitrofurazone was mixed directly with water omitting the ethanol step. After the crocodiles had been weighed and fed, the Nitrofurazone solution was then administered to the animals by pouring it over the visibly diseased parts of their bodies, including the buccal cavities when possible. The remainder of the solution was poured into the tank water for use as a subsequent immersion bath.

Glucose at a dose rate of 3 g/kg body weight was administered to the fourth crocodile on three separate occasions, each for several consecutive days. This followed the procedure as outlined in section 2.5.

Observations were carried out daily while the crocodiles were on campus. This usually occurred from 5 pm for approximately an hour, and on weekdays also

involved brief morning checks. A photographic record was made of various disease features. Mycological cultures were taken periodically following standard practices and post-mortems were carried out on each of the four observation crocodiles.

2.2 Histology

Hatchling carcasses were refrigerated until post mortems were carried out. Observations were made of any external manifestations of the disease. Skins were then removed following crocodile farming techniques and returned for salting by farm staff and for later tanning. Some carcasses were opened ventrally and viscera removed. The carcass with skin intact was then returned to the farm for skinning by farm staff. This not only saved time, but also allowed better skin removal without downgrading caused by damage due to worker inexperience. Post mortem procedures followed standard veterinary practice. Internal anatomical features were identified by referring to Chiasson (1962). Tissue samples were stored in neutral buffered formalin (NBF), and fixed for 24 to 48 hours. They were then processed in Tissue-tek process cassettes in an AO Histokinette Model E7326 automatic tissue processor prior to embedding in 56°C melting point Paraplast wax. The tissue was then blocked in Tissue-tek embedding rings at a Shandon Histocentre 2 tissue embedding station. Sections of 8μm were cut on a Leitz 1512 rotary microtome using steel knives, then floated out in Labmaster tissue flotation baths at 52°C to which slide adhesive had been added at the rate of 2 ml per litre. Individual sections were collected onto glass slides and dried overnight in a Labmaster incubator at 54°C. Slides were stained using Harris' Haematoxylin and Eosin (H & E) and also by the Periodic Acid Schiff Reaction (PAS). Both staining schedules followed the methods of Brown (1978).

Embryos were removed from the eggs following the method of Webb and Manolis (1987) and fixed in Bouin's Fixative for 48-72 hours. They were then washed in three changes of 70% ethanol to which lithium carbonate had been added to saturation, (~0.5g/100ml of 70% ethanol) to remove picric acid from the tissues. The embryos were then processed in the Histokinette from the 70% ethanol dehydration stage through to embedding following the same schedule as the NBF fixed tissue. Appendix 3 provides fixative and slide adhesive information and tissue processing schedules.

2.3 Mycology

Samples were routinely taken by scraping the surface to be tested with the wooden ends of sterile swab sticks. Swabbing with the cotton tips of these swab sticks resulted in cultures over-run with bacterial growth whereas scrapings which exposed the subsurface layer yielded predominantly fungal growth. Surfaces tested included walls and floors of incubation facilities and hatchling pens; freezer room shelves, walls and floor; and feeding trays, animal food preparation equipment and work benches. Scrapings were also taken from external skin lesions on the hatchlings and from the external surface of eggs. Culture tubes with slopes of half-strength Potato Dextrose Agar (HPDA) were used for primary cultures. These were incubated at 30°C and sub-cultured onto sterile HPDA plates or Sabouraud's Agar plates to obtain pure culture. Purified mycelial cultures from post-mortem or post-hatching tissue samples were obtained following the method of Harrower (1989a). Identification was carried out using Malt Extract Agar (MEA), Czapek Yeast Agar (CYA) and 25% Glycerol Nitrate Agar (G25N) following the method of Pitt and Hocking (1985). Slides were made at each sub-culture stage using Acid Fuchsin in Lactic Acid as the principal stain. Appendix 4 provides media and stain information.

Autopsies were also carried out at the Rockhampton Veterinary Laboratory at times of high hatchling and yearling morbidity. Various body organs were taken for histological and mycological investigation. Bacteriological tests were also performed.

Air settle plates were made in various areas of the farm. These were carried out inside incubation facilities, in the work shed at the animal food preparation area, inside the hatchling building, and outdoors along thoroughfares between the work areas.

Soil and sand samples were taken from various locations around the farm for subsequent mycological testing. Samples of natural nesting material for mycological testing were also collected from eight nests in the latter part of the 1990/1991 breeding season, and all nests in the 1991/1992 breeding season.

Water samples were taken from the farm well which was being used to supply water to hatchling facilities. Samples were taken from immediately below the water surface, from the outlet at the top of the well, and from the outlet at the hatchling building.

Sticky-tape samples were taken from various surfaces in the incubation facilities before and after cleaning with anti-fungal agents. Some of these tape sections were attached to microscope slides for immediate viewing. The remainder were placed on HPDA plates for mycological culture and subsequent identification.

2.3.1 Sensitivity Tests

Sensitivity tests performed early in the research program were aimed at finding a suitable treatment for the diseased animals. Sensitivity tests using *F. solani* had been performed on Nitrofurazone by Harrower (1989b) so these were not repeated. On request, additional sensitivity tests were carried out by Lord (1990) using *F. solani* and a range of common anti-fungal agents. Those selected for testing were amphotericin B, econazole, ciclopirox, griseofulvin, clotrimazole, ketoconazole, flurocytocine, isoconazole, tioconazole, natamycin, and nystatin. Tecto® (refer Appendix 1) with the active ingredient thiabendazole was also tested.

During 1991, laboratory based tests using standard mycological procedures were carried out to find a suitable antifungal agent with which to clean the newly laid eggs. A readily available and relatively inexpensive veterinary and agricultural product, Arocide®, was selected for testing. Another product tested was Virkon S®. This had previously been used in Zimbabwe to treat fungal infections affecting hide quality in juvenile crocodiles, including those infections caused by *Fusarium* sp., (Antec International Ltd. 1991). Malachite Green, an antimicrobial commonly used in the aquaculture industry to treat fungal infections in fish was also tested. Various other methods of treatment (*eg.* by formaldehyde vapour) were rejected due to the danger to humans and developing embryos. Appendix 1 provides information on Arocide®, Virkon S® and Malachite Green.

2.4 Haematology and Biochemistry

Six young crocodiles were physically restrained and transported by road from the crocodile farm to private premises in Rockhampton. The animals were wrapped loosely in hessian bags and rubber bands were used to keep their jaws closed. Three of the animals were apparently healthy three year olds (animals 1, 2, 3). The other three were twelve months old and were from a group which had ceased feeding and were

losing condition (animals 4, 5, 6). Others from that group had recently died from apparent fungal infection (Lever, pers. comm.). Animals 1 and 2 were female, animal 3 was male, however the other three were too small to sex properly. No weights or lengths were recorded. After the ventral surface of the tails was swabbed with alcohol, blood samples were taken ventrally from the caudal vein which runs along the ventral aspect of the coccygeal vertebrae (Samour *et al*, 1984). The needle was inserted between the scales in the ventral midline of the tail at approximately one third of the tail length (Samour *et al*, 1984). The needle was pointed slightly anteriorly and aspirated lightly as it approached the vertebra (Marcus, 1981). The blood was collected using eighteen gauge needles for the larger animals and twenty-one gauge needles for the smaller ones. Blood for haematological tests were collected in Exetainers which contained EDTA (ethylenediaminetetra-acetic acid, an anti-coagulant). Blood for biochemical tests was collected in Serum Separator Tubes (SST) with clot activator, (both tube types available commercially from reputable scientific suppliers). The samples were then despatched to the Rockhampton Pathology Laboratory at the Base Hospital for a haematological and full serum biochemical profile, with results available on the same day. Haematological tests were carried out using a Coulter Counter Model SPlus4. A Beckman Synchron CX5 Multi-channel Random Access Analyser was used for the biochemical tests. The whole blood samples were returned twenty-four hours later for haemoglobin quantification, red and white cell counts and smears for differential white cell counts. Haemoglobin tests were carried out on campus using a Superior Assistent Haemometer (24hr reading) and a HemoCue (72hr reading). Serum samples stored under refrigeration were also passed on to the Rockhampton Veterinary Laboratory for comparative and additional biochemical testing after six days.

2.5 Hypoglycaemia

Blood was collected in August 1992 from hatchling and yearling crocodiles suspected of suffering from hypoglycaemia. (Previous blood profiles were carried out in October 1990 to obtain healthy baseline and immuno-compromised readings.) Sampling was carried out on the farm at 8 am with animals captured and restrained by farm staff immediately prior to sampling. This enabled blood samples to be taken within ten

minutes of initial disturbance of the animals to avoid any changes in blood biochemistry due to stress. The three yearlings selected were all males approximately eighteen months old, with weight and overall lengths recorded. One was partially paralysed and suspected of suffering from hypoglycaemia. Two hatchlings, also possibly hypoglycaemic, were approximately five months old and were both too small to sex properly. Weight and length for these were not recorded. The ventral surface of the tail was swabbed with Tincture of Iodine (refer Appendix 1). Blood samples were then taken ventrally from the caudal vein following the previously used procedure (refer Section 2.4) using eighteen gauge needles for the yearling crocodiles. Due to the small hatchling sizes, blood was not able to be taken with twenty-one gauge needles. Blood for haematology was collected in tubes containing EDTA. Blood for glucose and other biochemical tests was collected in commercial tubes treated with fluoride oxalate. Additional tubes without anti-coagulant present were available for serum profiles. However insufficient blood was collected for these. All samples were transported to a veterinary pathology laboratory for a full biochemical (plasma) and haematological profile for each animal.

Immediately after blood was taken, the suspected hypoglycaemic animals were treated with glucose at the rate of 3 g/kg body weight as recommended by Wallach *et al* (1967). This was effected by dosing them with a concentrated glucose solution (refer Appendix 1), at 9 mls for the yearling and 1 ml each for the hatchlings. A miniature drenching gun was used for administering the solution. This was a 10 ml syringe with the nozzle aperture drilled out to allow easier liquid flow. To this was attached a 15 cm length of silicon tubing with an outside diameter of 4 mm and an internal diameter sufficiently small enough to allow a tight attachment to the syringe nozzle. The crocodile was held securely with its eyes covered and its jaw held open from behind the head with thumb and forefinger pressure on either side at the back of the jaw. The glucose solution was drawn into the assembled apparatus to fill both the syringe and tubing. The tube was then laterally inserted into the crocodile's mouth. Gentle pressure was needed to push through the palatal valve. The tube was then pushed down the oesophagus until it could be palpated in the area of the rib cage anterior to the stomach. The required dose was then slowly dispensed while at the same time the ventral surface of the crocodile's neck was massaged to stimulate swallowing. The

tubing was then withdrawn, the animal's jaws firmly closed and its neck further massaged to prevent regurgitation.

A second blood sample was taken from animal 1 in the early afternoon of the same day. The animal was recaptured, restrained and transported to the veterinary laboratory for this second blood letting exercise. All tests were carried out and results obtained on the day of blood collection.

2.6 Environmental Parameters

2.6.1 Temperature

At the start of the research program temperature in the A-frame (refer section 2.7.1) was recorded daily using simple maximum/minimum thermometers. A Hisamatsu battery operated thermohygrograph with paper chart (temperature range 0-50°C, humidity 0-100%) was run for weekly intervals and repositioned to new locations within the building at monthly intervals to cross check accuracy of manual recording methods and to check for possible hot or cold pockets. A Grant Squirrel Data Logger (Model 1201-20) with four temperature recording channels each with 5 m lead and probe (type CM-U-V5) and two humidity recording channels became available. It was used to record air and water temperature in various locations both in the A-frame and in the hot room and incubation room. Initially the data logger was programmed to record at twenty minute intervals over a week but this was subsequently changed to thirty minute intervals over a fortnight. Information was down loaded onto computer disk weekly or fortnightly via the farm computer for later analysis on campus.

During egg incubation an Ota spring-operated thermohygrograph with paper chart (temperature range 0-40°C, humidity 0-100%) was positioned on a shelf furthest from the doorway of the incubation room. This thermohygrograph was more accurate than that used in the A-frame but was similarly run for weekly intervals. A maximum/minimum thermometer was also located beside this instrument. A spirit thermometer (temperature range -10/110°C) was inserted into each tray of vermiculite in which the eggs were incubated. During egg incubation, the data logger with the four temperature probes was used exclusively in the incubation room. It was programmed to record temperature both in and out of the vermiculite at different incubation tray levels throughout the room using two of the probes. The location of

these two probes was varied after down loading. The internal egg temperature was recorded by inserting the third probe into an infertile egg then resealing the insertion site with nail varnish after checking for leakage. This egg was then housed in damp vermiculite in incubation trays similar in position to fertile developing eggs. The fourth probe was located beside another temperature recording device which was connected to a digital temperature display external to the room. This was in turn connected to an alarm bell and flashing light which were both activated at predetermined maximum and minimum settings.

2.6.2 Humidity

Humidity in the hot room, incubation room and A-frame was initially recorded daily by the use of wet and dry bulb thermometers. Readings were cross checked with a whirling hygrometer. The Hisamatsu thermohygrograph which was used to record temperature in the A-frame over weekly intervals also recorded humidity. A humidity probe (type VH-1-Z1) for the Grant data logger subsequently became available and was used in various locations to check the accuracy of the simpler recording methods. High humidity levels were maintained by the water levels in the tubs, tanks or pens.

During egg incubation the data logger and probe were used exclusively in the incubation room. Room humidity was recorded with the probe positioned to the right of and close to the door where greatest humidity fluctuations occurred due to staff entry and exit. Humidity levels within the damp vermiculite, similar in position to incubating eggs, were also recorded by the use of the data logger humidity probe. The probe was fitted into a ventilated PVC tube enshrouded by porous mesh to allow vapour cross flow but to prevent contamination of the probe sensor tip by the vermiculite. This assembly was buried in the vermiculite at the same depth as incubating eggs. The Ota thermohygrograph used in the incubation room for recording temperature also recorded humidity. High humidity levels were maintained by a trough of water which extended the full length of the room. Arocide® was added to the water to prevent bacterial growth. When the incubation room was used to house hatchlings, humidity levels were maintained by the water in the hatchling tanks or tubs.

2.6.3 Light Regimes

Light for the hot room and incubation room was provided by a single incandescent light which was turned on only while workers were present. Each room was otherwise in darkness.

Pens in the A-frame were lit by natural light through the translucent plastic skylights. For short periods daily these skylights allowed sunlight into parts of some pens to the right of the walkway. The raised pens on the left were quite dark because of their position under the roof.

2.7 Husbandry

2.7.1 Housing

New hatchlings were housed in rectangular plastic Nally tubs 34 cm wide, 52 cm long and 27 cm high, with a floor area of approximately 0.2m^2. Two or three 1 cm diameter drain holes were drilled in the tub base at one end, and the tubs were positioned with the drainage end raised by the use of a wooden support. This provided both wet and dry areas for the hatchlings, after cleaning and refilling with water. Later, Nally bin lids with a centre portion cut out (15 cm x 15 cm) were fitted to prevent the occasional escape of an active hatchling, but still allowing observations by staff.

Free-standing tanks positioned on shelves were purpose-built for housing hatchlings in the hot room or incubation room when the incubation season was finished. After the young hatchlings were observed to eat, generally between three and ten days old, they graduated from the Nally bins to these tanks. The tanks were made of sheet metal and were 2.2 m long, 1m wide with a depth of 10 cm. They were fitted with an A-frame cover to give a peak height of 22 cm. The fixed rear portion of this cover was made of sheet metal but the front was fitted with a hinged mesh grill to allow for observations and access. When some small hatchlings attempted to escape through the mesh, the mesh portion of the tank lid was covered initially with styrofoam sheets. These covers were later replaced with fitted shadecloth. A drain hole with external lever tap was fitted in the base at the centre of one end to allow easy drainage and cleaning. The tanks were positioned on supports such that the drainage end was slightly lower, thus also leaving a 50% dry area for the animals after refilling the tanks with water. An inverted and raised plastic frame (approximately 50 cm by 60 cm)

placed in each of the tanks to straddle both wet and dry areas, provided a safe haven or 'hide' for the animals. They were able to go both under and on top of the frame, which was also sufficiently low to prevent 'piling' and possible suffocation by some hatchlings when they huddle together when under stress. Being plastic, the frames were easily removed for cleaning and disinfecting or when counting or removing animals.

The first incubation room (known as the hot-room) was constructed of insulation panels for the walls and roof, and had a tiled concrete floor. The room was 2.92 m long, 3.05 m wide and 2.34 m high with a single door at the centre of one end.

The hot room was replaced by a second incubation room which was similarly constructed of insulation panels and concrete floor. This room was 2.67 m wide, 5.47 m long and 2.02 m high with a single doorway in the centre of one end.

Larger hatchlings and yearlings were housed in the A-frame, a building 16.77 m long, 3.45 m wide and 2.15 m high in the centre sloping to 1.20 m high at the sides. It was constructed with a concrete base, insulation panel walls and roof. The roof angles were offset from the peak to allow double thickness, translucent, vertical plastic skylights for the full length of the roof. The building was positioned to take the most advantage of the winter sun through these skylights. (PVC pipes were installed in the concrete foundations during construction of the incubation room and also the A-frame for future connection to solar heated water, to reduce future heating costs.)

At the commencement of research the A-frame was fitted out with floor pens only, to the left (total area 28.5 m^2) and right (total area 29.4 m^2) of a central walkway which was protected each side by upright walls 0.9 m high. These walls allowed entry to the pens by staff but prevented escape by hatchlings. Entry to and exit from the building was by one doorway only. This was installed with an additional inner lower mesh panel which prevented exit or entry of animals while still allowing air flow in summer when the main door was left open during the day. No windows were fitted to the building other than the vertical skylights at the roof peak. Floor area to either side of the walkway could be divided into from one to three pens (left side three pens each 9.5 m^2, right side three pens each 9.8 m^2) by the use of removable barricades. Each of these smaller pens was fitted with a central drain hole. The concrete floor was moulded during construction to allow filling the pens with water to a depth enabling

full submergence of the crocodiles in the deepest parts, and giving 50% water coverage and 50% dry area. Drainage of the pens was effected by the use of an external valve, while water supply was piped from the main storage tank to taps inside the A-frame. 'Hides' were provided on the floor by the use of sections of insulation panels raised off the floor by concrete blocks.

During the course of the research, additional pen space was required so new upper level pens were added to the A-frame. Five pens were constructed to the left of the walkway, each of the same size (5.7 m^2) and supported by a frame hanging down from the roof. The fronts of the pens aligned the walkway and were fitted on their upper halves with hinged mesh doors to allow cleaning, feeding, and removal or addition of animals. Each pen was fitted with a lever operated drain outlet, a raised dry area, and two plastic frames for 'hides' as used in smaller tanks. Two additional pens (each 3 m long, 1.55 m wide, area 4.65 m^2) were constructed to the right of the walkway, again suspended from the roof and with similar drainage conditions but of a more open construction to allow easier access and observation. Hatchlings in heated indoor pens can be seen in Plate 3b.

Additional tanks identical to those used in the incubation room were commissioned for use in the A-frame. These tanks were raised off the floor to the right of the walkway by the use of stacked concrete blocks and were situated either side of the two suspended pens. Since they were positioned over the floor pens, this made cleaning and observation more difficult in the lower pens.

At the completion of this research an additional A-frame was being constructed and plans were under way for other housing methods to allow easier access for cleaning, feeding and animal handling and incorporating better drainage, heating and ventilation design.

Animals not in research treatment or growth trials were assigned to various pens according to their size. Animals from the same clutch but with different growth rates were sorted visually at approximately three monthly intervals and relocated to new pens with other animals of similar size. Differing age groups were often housed together and were identified by the pattern of removal of their scutes (refer section 2.1 and 2.12). Variations in size due to different growth rates of three animals from the same clutch can be seen in Plate 4a.

2.7.2 Cleaning

At the start of the research hatchling and yearling facilities were cleaned daily using ambient temperature well water. This was later amended to twice daily, both before and after feeding, using chlorinated town water which had been pumped to a holding tank, and then heated before use. Heating was effected by a temperature-adjustable gas hot water system located at the storage tank outlet. The animal pens, tanks or tubs were partially refilled with water after cleaning to give a 50/50 dry-pen/water area and left overnight. Cleaning was by draining and then by hosing out while scrubbing with a stiff broom or brush. Glochlor bleach was used to clean the pens, and for soaking used food trays and implements. A solution of Arocide® was used to disinfect 'sick bays' before and after use, and before transferring animals between pens, or in any suspect areas. Arocide® was also used to disinfect the incubation room prior to use, including walls, ceiling and all fittings. A foot bath was installed at the entrance to the incubation room to minimise introduction of contaminants from the farm environment.

To prevent possible infection of the sometimes incompletely closed abdominal wall and unabsorbed yolk sac of newly hatched hatchlings, Tricon® (refer Appendix 1) was added to the water in the Nally tubs which housed them.

Aroclenz® (refer Appendix 1) a proprietary veterinary product, was used to assist in the cleaning and healing process of wounds and other skin lesions affecting the crocodiles. Its usage was by topical spray application, often without the need to handle the animals. The dark violet colour of the product also enabled easy visual identification of treated animals.

2.7.3 Food and Feeding

Food for the juvenile crocodiles at the commencement of this research (early 1990) consisted of lean meat fed daily, excluding Thursday and Sunday when staff were absent. This was sourced locally and variety depended on availability. It consisted of any one of the following - frozen then thawed kangaroo; fresh beef; fresh horse; fresh pig; fresh or frozen then thawed chicken. As a beast's fresh carcass became available it was sectioned into more easily handled portions and frozen.

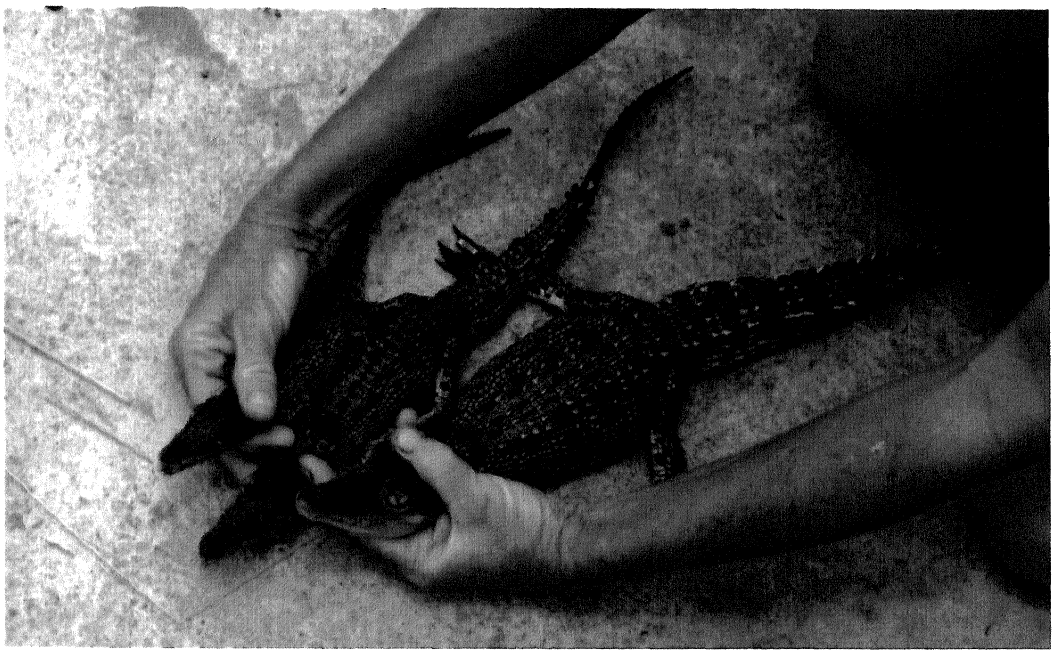

Plate 4a: *C. porosus* hatchlings from the same clutch showing variations in size.

Plate 4b: Healthy two year old *C. porosus* feeding.

As required, the meat was then thawed and manually cut into edible chunks of a size suitable for each age group to swallow easily. This size ranged from 1 cm cubes for the youngest hatchlings, through to 10 cm cubes for larger yearlings and for two year olds (refer Plate 4b).

Calcium di-phosphate and iodised salt were added to the meat although no other food supplements were used (Reimers, pers. comm.). A vitamin premix, Feramo D® (refer Appendix 2), had been included in previous years (Lever, pers. comm.).

The meat was apportioned by size to the various groups of juveniles, weighed, mineral supplements added, then placed on individual trays in each pen or tank. After an hour the tray and any uneaten meat was removed. This was also weighed and the amount consumed per group calculated. If all meat was eaten, more was allocated at the next feeding time, or the quantity reduced if uneaten food previously occurred.

In late 1990, a locally sourced vitamin and mineral supplement was added to the juveniles' diet regimen. Vita-Stress® (refer Appendix 2) was initially chosen. This was sprinkled over the meat along with the calcium di-phosphate and iodised salt and the combination mixed manually. For ease and continuity of supply, Pet-Vite® (refer Appendix 2) was later substituted and became the regular supplement.

In a growth trial Enzactiv® (refer Appendix 2, and section 2.11.3) was also added to the meat in combination with the other supplements.

Changes in the meat diet due to fluctuations in availability of supply caused a reduction in consumption for up to a week until the juveniles apparently became accustomed to the new taste and odour (Reimers, pers. comm.). In times of shortages horse and predominantly beef was allocated to hatchlings and yearlings, whereas pig, poultry and kangaroo meat was fed to older or larger juveniles more able to withstand temporary self enforced reductions in nutrient intake. Horse meat was consumed in the greatest quantities when it was available. Beef was the diet next preferred by the juveniles.

If staff time was available the meat was thrown onto the trays in the pens. This seemed to stimulate the juveniles to feed and more meat was eaten in this manner than if the loaded food tray was placed in the pen (Reimers, pers. comm.).

2.7.4 Heating and Cooling

Maxigrow Turbo Aire 2000W heater fans (thermostat controlled, temperature range 5-40°C) as used in the horticulture industry, were used for heating. One was located in the incubation room and two were positioned at opposite ends of the A-frame. A household oil heater was also used to provide additional warmth in the A-frame during winter. Cooling in summer was effected by electric fans when necessary combined with natural ventilation through an opened stable-type doorway.

2.7.5 Personnel

Access to the incubation facilities and to hatchling and yearling rearing pens in the A-frame was restricted to one permanent farm employee, farm management and the researcher. This ensured continuity of data recording and reduction of operator error due to inexperience. This also reduced the possibility of contamination being introduced by workers. The incubation and rearing facilities were located away from areas accessed by tourists.

2.8 Egg Incubation

2.8.1 1990/1991 and Previous Breeding Seasons

Up to and including the 1990/1991 breeding season, (egg-laying generally from November to March, hatching from February to late May) eggs were collected from the nests and incubated without any treatment, *ie.*, no washing or dipping or the use of any other decontamination process. The first incubator used at the farm was a simple, small, chicken egg incubator. In later years, this was replaced by a small laboratory incubator with open racking. By 1990/1991 this had been replaced by a purpose-built large shelved incubator also with open racking. Temperature control for this last incubator was achieved by a thermostatically controlled fan heater, monitored with a digital thermometer. High humidity levels were maintained by a large tray of water in the base of the incubator. The incubating eggs could be visually inspected through the acrylic doors. Infertile eggs, and fertile eggs which had ceased development were removed when necessary.

Of twenty-six clutches of eggs incubated in the 1990/1991 breeding season, eight clutches in the latter part of the season (April and May of 1991) were tested for fungal

infection. Samples were taken from the shells, shell membranes, infertile eggs, early embryonic discs, the extra-embryonic membranes immediately after hatchling emergence, and from dead embryos. The air spaces of some unhatched full term eggs were also sampled. Samples of nesting material were taken from one nest for subsequent mycological testing.

2.8.2 1991/1992 Breeding Season

During the 1991/1992 and 1992/1993 breeding seasons investigations were carried out to determine an effective way to remove fungal propagules from freshly laid eggs without damaging the developing embryo. Also, investigations were initiated to determine the method of entry of the pathogens through the shell. The oviducts from a breeding female, killed by another crocodile while defending her nest, also became available and were sampled for mycological culture. An adult female *C. porosus* guarding her nest is shown in Plate 5a.

Eggs were collected from the nests for artificial incubation. Immediately prior to removal from the nest (refer to Plate 5b), the top surface of each egg was marked in order to maintain the correct orientation for future development of the embryo (Webb *et al,* 1987). The oviducal mucous layer on the surface of each egg and any adherent nesting material were then washed off by hand using running tap water at ambient temperature. This temperature ranged from 20°C to 27°C. The washed eggs were then dipped in a bucket of Arocide® diluted in tap water at a ratio of 1:150 for 5 minutes. This involved placing the eggs in a plastic bucket which had holes drilled in its walls and base, and submersing this bucket inside a larger one already filled with the antifungal agent. After the treatment time, the bucket of eggs was lifted out and allowed to drain. The cleaned and decontaminated eggs were then positioned in trays of dry vermiculite on racks in a walk-in purpose-built incubation room. A thermostatically controlled electric fan heater was used to control temperature and two large troughs of tap water helped maintain high humidity levels. Temperature and humidity were monitored using a chart recording thermohygrograph, and a Grant Data Logger with four temperature probes and one humidity probe.

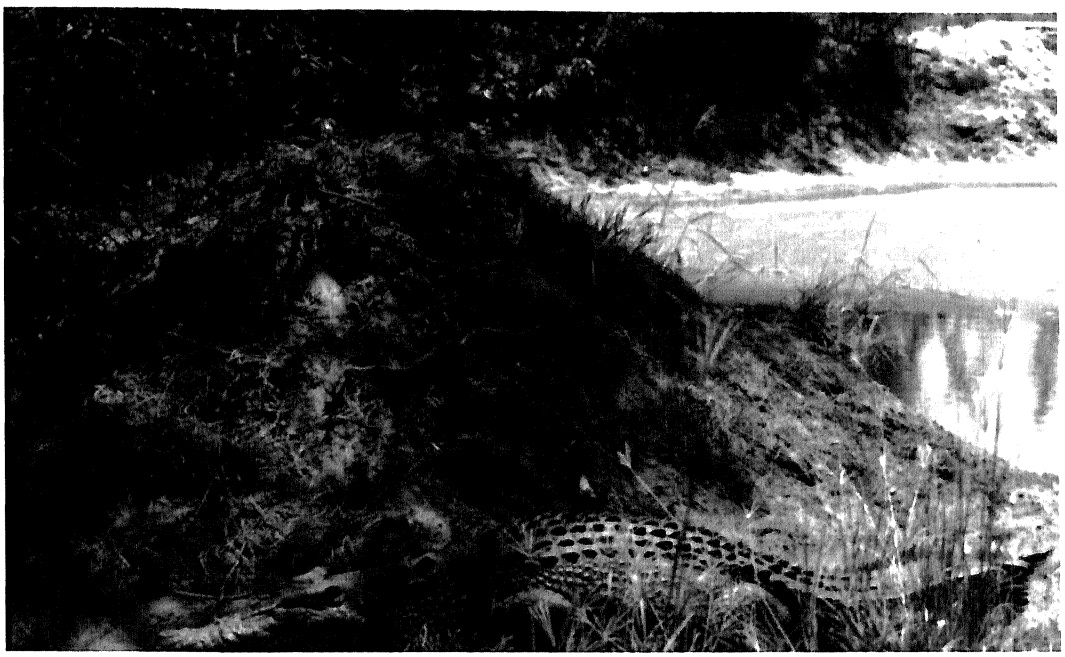

Plate 5a: Adult female *C. porosus* guarding her nest.

Plate 5b: *C. porosus* eggs in nest, uncovered prior to collection.

Nesting material from all nests was collected in the 1991/1992 breeding season and sampled mycologically again using standard procedures. The eggs were also randomly sampled before washing, after washing, after dipping, and during incubation.

2.8.3 1992/1993 Breeding Season

The procedures and materials used were as for the previous year, except that the incubating eggs were positioned in trays containing wet vermiculite, moistened until droplets of water could be squeezed out (Blake, 1991). This was trialed after discussions with Zimbabwean and South African crocodile farmers and researchers during the 11th Working Meeting of the Crocodile Specialist Group in August 1992.

Later in the season, the vermiculite was dried out until it was damp but not as moist as previously. Eggs were sampled mycologically after washing and after dipping. Eggs were also candled after one weeks incubation and infertile eggs removed. Those with embryos which subsequently died were removed at regular intervals for examination and mycological culture.

Incubating eggs, showing banding development and hence their viability, are shown in Plate 6a. A humidity probe became available for use with the Grant Data-Logger and was added to the other environmental monitoring equipment used previously.

2.8.4 1993/1994 Breeding Season

The 1993/1994 breeding season involved further refinement of the egg decontamination treatment.

The process was modified in that eggs were no longer washed by hand using ambient temperature tap water, but were irrigated by warm tap water using hose attachments.

Water was heated by a temperature-adjustable gas hot water system positioned at the water storage tank outlet. This allowed water temperature at the outlet nozzle to be approximately 3C° higher than that of the recorded nest temperature at egg collection.

The partially treated eggs were then dipped for 5 minutes in the Arocide® solution (1:150) at the same temperature as the washing water and then incubated as previously in damp vermiculite. Candling and removal of non-viable eggs followed the procedures of the previous year.

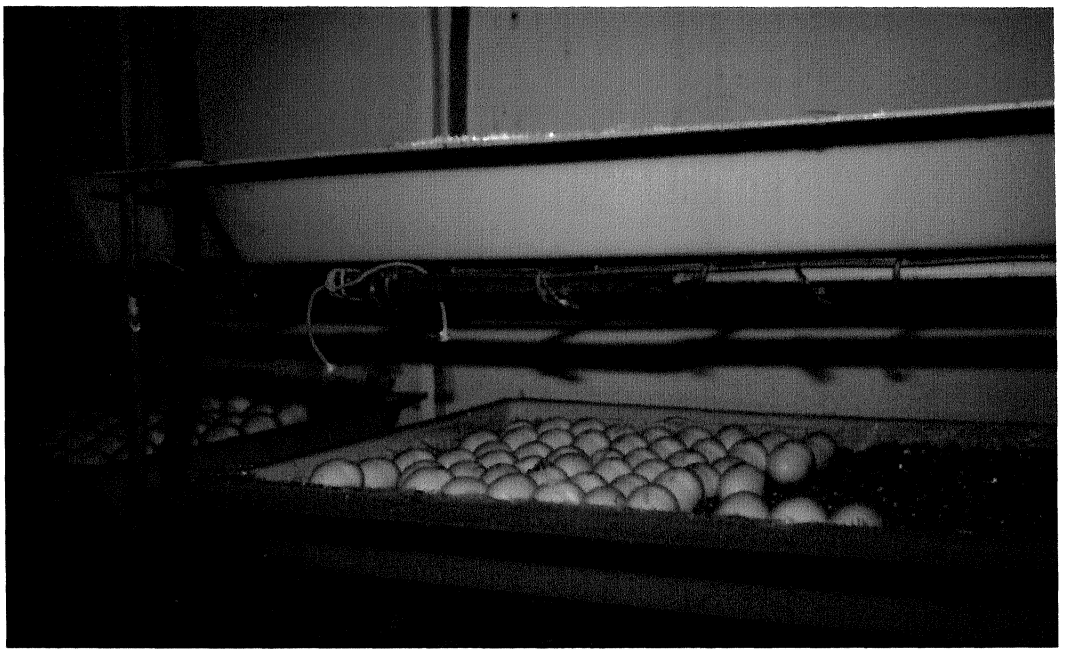

Plate 6a: Incubating eggs showing banding.

Plate 6b: *C. porosus* hatchling emerging from its egg.

After the emergence of hatchlings (as shown in Plate 6b) any remaining unhatched viable eggs were carefully assisted in their hatching process, or those with full term but dead embryos were examined for defects and processed for mycological culture.

2.9 Photography

A Canon AE-1 Program camera with a wide range of accessories was used for photography. Accessory items used were Canon 50 mm macro lens and extension tube; Canon 35-70 mm zoom with macro; Tokina 50-250 mm zoom with macro; Canon 100-300 mm zoom with macro; and a Vivitar hot-shoe flash and Toshiba external flash. A connector tube and t-piece enabled camera attachment to an Olympus SZIII trinocular zoom stereo microscope and also to an Olympus CHA trinocular compound microscope. This collection of photographic equipment enabled the use of one camera body in all situations from long distance to close up macro-photography both indoors and outdoors. Low power photomicrography from x7 to x40 magnification as well as high power photomicrography from x40 to x1000 magnification was achieved. Kodak Ektachrome 100HC colour slide film was used for all colour photography and was processed commercially. Kodak T-Max 100 film was used for black and white photography, and was developed in Biology Department dark room facilities following standard procedures. Black and white prints were processed at CQU by Educational Media Section staff using their photographic facilities. Computer scanning and final printing of all photographs as presented in this thesis were carried out by staff of the Educational Media Section at CQU. Unless otherwise acknowledged, all photographs were taken by the author.

2.10 Scanning Electron Microscopy

Shells from both infertile and fertile eggs of *C. porosus* were collected during incubation and at hatching. Samples were taken for culture following standard mycological procedures. Randomly selected segments of shell (less than 1cm^2) were stored in 70% ethanol. These were later air dried, attached to aluminium stubs using adhesive tabs and silver DAG, then gold coated to a thickness of 15 nm in a Polaron SC515 Evaporation Plant. These were examined using a Jeol JSM-5300LV Scanning

Electron Microscope in high vacuum mode, with secondary electron images recorded on 35 mm Kodak T-Max black and white film.

2.11 Trials

2.11.1 Nitrofurazone Treatment Trial

Soon after commencement of this research and following a period of high hatchling mortality at the farm, a small trial was established (August to October 1990) to investigate the effect of Nitrofurazone on obviously diseased hatchlings. This product was selected based on results of sensitivity tests using *F. solani* on Nitrofurazone carried out by Harrower (1989b). Nitrofurazone had also been used successfully to combat fungal infections in fish in the Aquaculture Unit on the Central Queensland University campus, and the product was readily and cheaply available. The trial at the farm was run concurrently with treatment of two diseased yearling crocodiles on campus.

The hatchlings were housed in each of two hatchling tanks as previously used in the hot room. These tanks were set up in the A-frame and were cleaned and sterilised prior to use by soaking and scrubbing with Glochlor bleach solution. Except for the Nitrofurazone treatment all other husbandry methods followed standard farm procedures.

Due to low numbers of animals available, only two groups were able to be selected, Group A to be treated daily and Group B with no treatment. Each group had seven hatchlings all of which were obviously diseased with visible lesions along the upper and lower jaw line, around the eyes and external nares, and on the feet and torso. Other disease manifestations were loose and missing teeth, poor body condition, and depressed appetite.

Attempts were made to select crocodiles of similar size, age and genetic origin such that both groups were as similar as possible. Close similarity could not be fully implemented due to small numbers of stock available and the then method of crocodile identification by investment group rather than by clutch. Composition of each trial group may be referred to in Table 8.

After selection each animal was individually tagged with a size three monel metal tag (National Jiffy Style 898 Wing Band) obtained from the Queensland National Parks and Wildlife Service. Each tag, coded with a four digit number, was fitted to the inter-

digital webbing between the third and fourth metatarsals of each crocodile's right rear limb. After tagging each animal was weighed and measured using the same equipment and procedures as those used on campus. All surviving animals in both groups were reweighed on a weekly basis for a period of eight weeks, whereas each individual's length was only remeasured after four weeks. After any animal's death, its weight and length was again recorded.

Trial Group	Source Investment Group	Trial Group	Source Investment Group
A	1	B	3
A	6	B	3
A	6	B	6
A	7	B	6
A	7	B	7
A	8	B	8
A	11	B	11
Total Group A	7	Total Group B	7

Table 8: Composition of Groups for Nitrofurazone Treatment Trials.

Treatment of Group A crocodiles was effected daily by their full immersion in a bath of Nitrofurazone. Nitrofurazone was pre-measured into vials on campus such that its addition to the correct volume of warm water in the hatchling tanks would result in a solution with a final concentration of 10 mg/l. The hatchlings were fully immersed in this solution after the tanks had been emptied, cleaned and refilled. Repeated treatments occurred when the animals moved into or out of the water to regulate their body temperature. As this treated water was the only source for drinking by the hatchlings, they also ingested unknown small volumes of Nitrofurazone.

Ambient air temperature and water temperature was monitored on a daily basis as were observations on the animals' body condition. Overall food intake for each group was also recorded. Scrapings for mycological culture were taken from lesions on the animals which were selected at random from each treatment group. Post-mortems were carried out on those animals which died during the trial.

2.11.2 Thiabendazole Treatment Trial

A treatment trial using thiabendazole (commercially available as Tecto®) was established in May 1991 after the completion of all incubation and hatching for that current breeding season. This treatment trial was carried out in conjunction with two other growth trials. The first compared the effect of use or absence of food supplements in the hatchlings' diet on growth rates to check for nutritional stress. The second compared the effect of different animal numbers in the hatchling tanks on growth rates to check for density stress.

For these combined trials the old hot room was used. It was stripped of all incubation fittings, thoroughly cleaned then fitted out with seven hatchling tanks. These were supported in frames so that three tanks were stacked above and equidistant from each other on the left side of the room, and four were similarly tiered on the right side of the room. All groups in the room were housed at the same air and water temperature and humidity which were recorded as described in section 2.6. Husbandry methods for all groups were identical and followed those procedures detailed in section 2.7. Commercially available frozen kangaroo meat was used as the food for all groups. This meat was thawed daily prior to the addition of the various supplements and total food intake for each group was recorded.

A total of one hundred and fifty hatchlings were selected while they could still be identified as coming from a particular clutch. Hatchlings from clutches one to six were already mixed as one group as were those from clutches seven and eight. The hatchlings selected were tagged with monel metal tags following the procedure used in section 2.11.1 (Nitrofurazone trial). The tagged hatchlings were then weighed and measured and assigned to the seven different treatment tanks such that a representative mix of animals from different available clutches was established. The initial composition of all trial groups is summarised in Table 9. All hatchlings in these trials were weighed and measured at monthly intervals for a period of six months after which the trial ceased.

At the start of this trial thiabendazole was initially administered to the relevant hatchlings by fluid drench. A modified Phillips Model 74 Automatic Injector was used to effect this. The drench was made up as a water based suspension which when dosed to the hatchlings gave 50 mg of thiabendazole per kilogram of hatchling body

weight. This ensured all relevant animals received their initial dose and thereafter the treatment was administered by addition to their daily food supply. The correct weight of thiabendazole powder was sprinkled over the meat for that group after calculating the group's total body mass and projected food intake. The groups which received thiabendazole also received the full complement of dietary supplements, *ie.* calcium di-phosphate, Vita-Stress® and Enzactiv®.

Original clutch	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
1-6	2	2	2	1	5	2	2
7-8	3	3	3	1	5	3	3
9	3	3	3	2	5	3	3
10	3	3	3	1	5	3	3
11	1	1	1	1	2	1	1
12	6	6	6	3	15	7	6
14	2	2	2	1	3	1	2
Total →	20	20	20	10	40	20	20

Table 9: Composition of groups for thiabendazole treatment, food supplements and stocking density trials. (Data shows number of individuals per group)

2.11.3 Food Supplements Growth Trial

Basic food rations of kangaroo meat with added calcium di-phosphate were considered to be the minimum requirements for growth (Lever, pers. comm.). This became the sole diet for Group 1 as well as the base formulation for all of the other groups diets. Additional vitamins were added to the meat for Group 2 using Vita-Stress®. Group 3 also received Enzactiv®, which included a wide range of amino acids, vitamins and minerals, as well as additional crude protein, fat, fibre, ash and water. The supplements at the recommended rate were added to the meat similarly to the method used for the addition of thiabendazole, in that the supplements were sprinkled over the meat for the relevant group after calculating the group's total body mass and projected food intake. Groups 1, 2 and 3 were all held at the same density of

twenty hatchlings per tank. None of those three groups were administered thiabendazole.

2.11.4 Stocking Density Growth Trial

Density stress and its effect on growth were to be tested by comparing growth rates of groups of ten, twenty and forty hatchlings held in three tanks of identical size. These groups all received the full complement of food supplements (calcium di-phosphate, Vita-Stress® and Enzactiv®) as well as the thiabendazole treatment.

In these series of trials the following were to be compared:-

1. nutritional stress between groups 1, 2 and 3,
2. density stress between groups 4, 5, 6 and 7,
3. thiabendazole treatment between groups 3, 6 and 7.

Table 10 summarises the groups and the relevant treatments, supplements and densities. Group 7 was a replicate of group 6.

Treatment ↓ Group →	1	2	3	4	5	6	7
Kangaroo meat	✓	✓	✓	✓	✓	✓	✓
Plus calcium di-phosphate	✓	✓	✓	✓	✓	✓	✓
Plus Vita-Stress®		✓	✓	✓	✓	✓	✓
Plus Enzactiv®			✓	✓	✓	✓	✓
Plus thiabendazole				✓	✓	✓	✓
Density 10 per tank				✓			
Density 20 per tank	✓	✓	✓			✓	✓
Density 40 per tank					✓		

Table 10: Treatments, Supplements and Densities for effect on Growth.

2.11.5 Light/Darkness Growth Trial

To ascertain whether sunlight or darkness affected the onset of fungal disease in hatchlings or affected hatchling growth rates, a trial was established in October 1991 using crocodiles hatched in that year. Sixty hatchlings were selected and graded according to size into two groups of thirty, subsequently labelled as large and medium. Each of these two groups were then randomly subdivided into two groups of

fifteen. Clutch origin was noted. However, hatchlings were not allocated to groups according to clutch. One group each of large and medium were placed in upper level pens to the left of the walkway in the A-frame. The position of the pens under the ceiling ensured that they did not receive any sunlight and that they were sufficiently dark such that a torch was required to enable their inspection even at midday. The other two groups were housed in the upper level pens to the right of the walkway in the A-frame. These pens received high levels of light through the skylights and for a short part of the day could receive direct sunlight. Pen shape and thus area available was slightly different for the dark pens. Identical 'hides' were provided for all four groups. Food and dietary supplements were identical for all groups as were husbandry procedures (refer section 2.7). Air and water temperature and humidity for all four groups were monitored daily using equipment described in section 2.6. At the commencement of the trial all the crocodiles were tagged, then weighed and measured. They were also weighed and measured at monthly intervals for the subsequent three months after which the trial was terminated.

2.12 Egg and Hatchling Survival and Development

Historical data for egg production for the years 1984 to 1991 inclusive were accessed from the farm records. For the period 1992 to 1994 inclusive, data were collected as part of this research program.

Basic information recorded by clutch for the 1991/1992 breeding season was:-

- the total number of eggs laid (and collected for artificial incubation),
- the number of eggs damaged during or after laying and determined as not suitable for incubation,
- infertile eggs (removed from the incubator one to two weeks after setting),
- embryonic deaths (eggs removed from incubator after development ceased), and
- live hatchlings, and numbers scuted.

Additional information recorded was date of being laid (or date of collection), date of hatching, male and female parentage, nest location, whether or not nesting material was sampled mycologically, the condition of the eggs at collection, details of cleaning and disinfecting treatment and sampling of the eggs, which person processed the

clutch through to incubation, and any physical oddities or defects in dead embryos or hatchlings.

During the 1992/1993 breeding season and later, in addition to the above, nett weight of eggs set by clutch was recorded.

Attempts were made to determine possible cause of embryonic mortality. Each egg was individually weighed, and its length and diameter measured. The stage of banding of each egg and the position of each embryo in its egg relative to the uppermost surface were then recorded. Eggs were then sampled mycologically followed by processing of each embryo for possible future histological investigation. The size of each embryo was also recorded so that developmental age could be determined.

Historical data for hatchling survival for 1989 (and prior) were accessed from the farm records. For the period 1990 to 1994 inclusive data were collected as part of this research program. Basic information recorded by clutch was number of live hatchlings produced at the end of each year's breeding season and the number of survivors as at 31 December of the corresponding year.

Weight of individuals from each clutch after hatching was recorded by weighing all of the live hatchlings as a group then calculating the mean.

From and including 1992 all hatchlings were identified by scuting as for previous years. However, the scutes were removed according to clutch number not investment group. The procedure followed that detailed in section 2.1. Additionally the year of hatching of each animal was marked by removing a single scute from the single row of scutes along the animal's tail. From the Y-junction, and counting posteriorly, each scute designated the year starting from zero to nine, thus 1992 was identified by the removal of the third scute, 1993 by the removal of the fourth and so on.

On 31 December yearly all hatchlings were counted, their clutch origin noted, then they were weighed and their overall body length measured. At that time they were also graded and allocated according to size to new pens. In 1993 this procedure was also carried out on the 30 June.

2.13 Koch's Postulates

To validate Koch's Postulates two sets of experimental infections were devised, one involving developing embryos in incubated eggs and the other involving new hatchlings. Laboratory procedures were similar to those of Sinclair and El-Tobshy (1969) and Booth (1971b).

A series of slope lawn cultures of *F. solani* was established in sterile culture tubes using HPDA as the growth medium. These were subcultured until multiple freely-sporulating cultures of the same age were produced. The conidia were harvested by initially adding 9 mls of sterile distilled water and a drop of Tween 80 to each culture tube, then using a combination of vortex mixing and rubbing the colony surface with a sterile blunt probe to loosen the conidia. The conidial suspensions were then poured off and collected into one sterile container. A conidial count was carried out using a haemocytometer following the method of Booth (1971b), and the suspension diluted with sterile distilled water to yield a final count of viable conidia of approximately 10^6/ml. Centrifugation was to have been used if the conidial count was too low.

The suspension was then divided into two equal volumes, one of which was autoclaved, thus resulting in both viable and non viable suspensions. Duplicate test cultures from each suspension were cultured to confirm their viability. Both viable and non-viable suspensions were used in order that both sets of eggs or hatchlings in each group were subjected to the same physical trauma. After completion of all procedures a second set of duplicate test plates were cultured using the 'used' suspensions to confirm the viability of the conidia.

Eggs with a known stage of embryonic development were selected for experimental infection (embryonic age sixty days, expected hatching age eighty five days). All were from the same clutch to minimise genetic variation (1993, clutch 40). These eggs had been processed following the procedures outlined in section 2.8.3, and had developed normally during incubation. Eighteen eggs were allocated to this trial with the remainder of the clutch left undisturbed to complete the incubation process. All eggs were coded to identify the treatments and were allocated to treatments as summarised in Table 11.

Treatment ↓ Inoculum→	Viable conidia	Non-viable conidia
Injection, 0.4 ml	3 eggs	3 eggs
Dip 5 minutes, cool (~27°C)	3 eggs	3 eggs
Dip 5 minutes, warm (~32°C)	3 eggs	3 eggs

Table 11: Experimental Infection of Eggs to validate Koch's Postulates.

Injection was carried out to ensure penetration of the egg by the inoculum and thus to determine its effect on the developing embryo. Dipping was carried out to test the theory that conidial penetration was effected either during cooling of the egg while in the nest, or during the egg treatment process when using ambient temperature water to wash the egg. The dipping time of five minutes was selected as this corresponded with the dipping time as used during the initial treatment with Arocide® prior to incubation. The cool dip temperature of *ca.* 27°C (actual 27.3°C) was selected as this corresponded with the tap water temperature most frequently measured during the egg washing stage. The warm dip temperature of *ca.* 32°C (actual 32.4°C) corresponded to the temperature most frequently encountered in the vermiculite surrounding the incubating eggs. Temperature was measured using a Grant Data Logger. Dipping was carried out in glass containers (histological staining dishes) which were slightly larger than the eggs. The temperature was regulated by filling these containers with the relevant suspension then placing them inside larger baths of water which had previously been allowed to equilibrate to the required temperatures (tap water bath or incubator water bath). After dipping the eggs were returned to the incubation trays.

Prior to injection the eggs were candled to determine the position of the embryo and any air spaces. The surface of each egg was then swabbed using cotton wool and disinfectant (aqueous potassium permanganate 1:500, 0.2gm/100ml). A small hole was punched through one pole of each egg into the egg albumen using a twenty-five gauge needle, then 0.4 ml of inoculum was injected into the egg. The needle was then withdrawn and the hole sealed with quick-drying nail varnish. When it was determined that no leakage had occurred each egg was then returned to the incubation tray. An unused sterile needle and syringe was used to inject each egg.

Twelve hatchlings of a known age and of a single genetic origin (1993, clutch 27, three days old) were selected for experimental infection. All were of similar size (mean body length 305 mm, mean weight 63 g) and appeared to be normally developed and free from external manifestations of disease. After treatment the animals were housed in groups of three in separate Nally Tubs, with all husbandry procedures following standard practices for that year. Table 12 summarises the treatments.

Treatment ↓ Inoculum→	Viable conidia	Non-viable conidia
Interperitoneal injection, 0.2 ml	3 hatchlings	3 hatchlings
Inhalation, 0.2 ml	3 hatchlings	3 hatchlings

Table 12: Experimental Infection of Hatchlings to validate Koch's Postulates.

Prior to injection, each hatchling's abdomen was swabbed with the potassium permanganate solution. Using sterile syringes fitted with a twenty-five gauge needles, 0.2 ml of inoculum was injected into each hatchling's interperitoneal cavity, after first lifting the skin and body wall and holding it away from the body proper, and taking care not to lacerate internal organs. All animals were injected into the same area (ventral, right side of abdomen, immediately posterior to the thoracic ribs).

Intranasal inhalation was effected by first clamping each hatchling's jaw closed with rubber bands, then using a syringe and twenty-five gauge needle to place several drops of inoculum onto the external nares. The animal was then allowed to inhale before the next drops were added and this process continued until 0.2 ml had been inhaled.

3. RESULTS

3.1 Preliminary Laboratory Observations

Of the three yearling crocodiles initially transferred to CQU campus in order to make daily observations on the progress of the disease, two died (crocodiles 6.1 and 10.1) within the first twenty-four hours. Neither accepted food, both defecated once, and both suffered a loss in body weight (crocodile 6.1 initial 208 g, final 200 g, loss of 3.8%; crocodile 10.1 initial 458 g, final 440 g, loss of 3.9%). Crocodile 6.1 was extremely weak and lethargic on arrival, appeared close to death, and barely changed position up to its demise. Crocodile 10.1, in contrast, appeared extremely agitated. Symptoms were very similar to those in captive alligators as described by Wallach *et al* (1967) and, therefore, a tentative unproven diagnosis of hypoglycaemic shock was made. The crocodile had pronounced dilation of the pupils (mydriasis) and repeatedly arched its head upwards and backwards (torticollis, described by Wallach *et al* (1967) as 'star gazing'). This later deteriorated into violent body tremors with the crocodile carrying out 'death rolls' similar to that of adult crocodiles when attacking their prey. During these rolls the crocodile's whole body arched (opisthotonos) and when this occurred in the water, it appeared to be in danger of drowning due to the apparent muscle paralysis. When removed from the water, the crocodile went into a state of deeper paralytic shock (catatonia) from which it did not fully withdraw, later reverting to body spasms then repeating the cycle.

The third animal (crocodile 11.1) survived for eleven days. Its initial weight of 436 g rose by 64 g to 500 g within two days (14.7% increase). However, this was followed by a steady weight loss up to its death, with a final weight of 414 g. When calculated from arrival weight this loss was 5.0%. However, if calculated from maximum weight the loss was 17.2%. Defecation occurred daily for the first three days, ceased for the next four days then recurred daily until death. Diced fresh beef was offered on day two but the crocodile did not eat voluntarily. It was then force fed daily with diced fresh beef on days three to six when partial regurgitation occurred. Force feeding using beef was again tried on day nine, again with subsequent partial regurgitation. On day ten, 5 ml of beef and vegetable pureed baby food was administered successfully. This crocodile was also treated with Nitrofurazone (initially dissolved in ethanol then diluted in water to give a final observation tank dilution of 10 mg/l), by

pouring the solution over the visibly diseased parts of the body including into the buccal cavity, and subsequently by immersion in the observation tank water. The crocodile became agitated during and after this treatment. The Nitrofurazone did not appear to assist in the healing of any external body or buccal cavity lesions.

The fourth crocodile, (11.2) arrived two days before the death of crocodile 11.1. Arrival weight was 368 g and, similar to crocodile 11.1, this increased by 64 g to 432 g within two days (17.4% increase). Over the next ten days there was a daily weight loss to a low of 390 g, followed by a steady increase to a new maximum of 420 g by day twenty-eight. This was followed by an overall gradual daily decrease over the next sixty-five days until the death of this crocodile after ninety-three days on campus. Total weight loss from arrival to death was 17.4%, or if calculated from maximum weight was 14.8%. Evidence of defecation was observed daily. Nitrofurazone initially made up in ethanol was administered daily for the first eight days after which the preparation stage using ethanol was omitted and a water based solution was used. After the first thirty days when the crocodile appeared to be progressing well, the Nitrofurazone treatment was reduced to alternate days only for two weeks, then ceased for two weeks. It was reintroduced when weight loss became evident. As observed with the previous crocodile, the Nitrofurazone did not appear to assist in the healing of any external body or buccal cavity lesions.

Fresh lean beef steak and kidney, or calf liver, diced into pieces of up to 1 cm^3 with added food supplements of iodised salt and calcium di-phosphate were fed to this fourth crocodile. Although it did not feed until day two it was, however, strong enough to commence feeding voluntarily. Weighed food was presented daily with approximately 6 g to 7 g consumed each day. After thirty days, feeding the crocodile on alternate days was tried, with 8 g to 14 g being consumed on each occasion. After a further two weeks, daily feeding was recommenced with amounts consumed varying between 5.5 g and 11 g per day. Evidence of regurgitation was not observed until after the alternate daily feeding was commenced. Even so, regurgitation in small amounts was only evident after relatively large quantities of food had been consumed on each occasion and this was not considered a major problem.

Glucose at a dose rate of 3 g/kg body weight was administered to this crocodile daily from days nine to thirteen inclusive. For the first nine days the animal had been

subjected to daily visits by unauthorised persons and became highly agitated with symptoms of mydriasis and body tremors similar to that observed in crocodile 11.1. After receiving each glucose dose, the crocodile almost immediately became calmer, with muscle relaxation and cessation of body tremors observed within two to three minutes. At around sixty days after similar agitation of unknown cause was observed in this crocodile, glucose treatment was again administered with a similar calming effect. As for crocodile 11.1, a tentative unproven diagnosis of hypoglycaemic shock was made in each of these instances. After relocating the observation tank, all associated equipment and the crocodile to a more secure and quieter location two weeks before its death, dosing it with glucose was effected as a precautionary measure in anticipation of a possible bad reaction to the stress involved. No hypoglycaemic symptoms were subsequently observed.

Humidity in the closed room at time of daily recording varied from 51% to 96% with the vast majority of readings being in the 80% to 90% range. The low humidity levels were usually recorded after someone had entered or left the room.

Room air temperature varied over the range 25-30°C for the first month, after which settings were adjusted to increase the temperature range to 26-32°C. Observation tank air temperature was recorded only for the last two weeks of observations and ranged from minimum of 26°C to maximum of 33°C. Observation tank water temperature initially varied between 26-33°C. This was later adjusted to between 27-34°C. Fluctuations in the water flow during calibration and initial use of the observation tank caused minimums as low as 22°C if flow was too fast for the heating capacity, or maximums of up to 35°C when flow stopped completely.

Crocodiles 11.1 and 11.2 preferred to either bask on the rocks near the infra-red lamp or rest over the aquarium heater's cover thus indicating a preference for the warmest areas of the tank. If disturbed the animals retreated to the deep water underneath the ramp until all noise and vibrations in the room had ceased.

3.1.1 Disease Manifestations

Observations by Koorana Crocodile Farm staff (Lever, pers. comm.) in 1988 were that the disease was initially apparent as a slimy covering on the back of one of the hatchlings. That animal died the following day. Scrapes and samples taken from that

animal were identified as *Fusarium* sp. (Hunt et al, 1988) and further identified by Harrower (1988) as being *F. solani*. Other animals showed similar signs of fungal infection of the skin. Histological re-examination (using mycological stains) of tissues taken from juvenile crocodiles which had died in 1986 from unexplained causes (Hunt and Aiken, 1986) showed evidence of invasive fungal hyphae.

The fungus manifested itself on the crocodiles' bodies as off-white to yellowish lesions along the upper and lower jaws, around and between the teeth, and within the buccal cavities (refer Plate 7a). The affected animals' teeth became quite loose and frequently fell out or dislodged easily. The ventral surfaces of the lower jaws were also severely affected (refer Plate 7b), as were the external nares and around the eyes. This included infection of the surface of the nictitating membrane. The toes and ventral surfaces of the feet were also affected by widespread necrotic lesions with, in severe cases, the infected skin sloughing off (refer Plate 8a) and claws being dislodged. Off-white to yellow fungal lesions also appeared between the scales along the top of the head and the back as well as between the scales on the belly skin. The dorsal scales became covered with a dark grey slimy material and often sloughed off by themselves, or lifted off during handling of the animals (refer Plate 8b). Lesions also were found on the skin at the leg joints. The slight violet coloration of the animal's skin in Plate 8b was due to the prior application of Aroclenz®.

Internally the infection manifested most frequently as multiple or single yellow granulomatous inflammations below the capsular surface of the liver (refer Plates 9a and 9b), and multiple or single small or large inflammatory foci in the lung. Other organs which appeared affected were the spleen (yellowish caseous foci), and the stomach, small intestine and the bowel (ulcerations on the walls). The livers and spleens often appeared enlarged. The kidneys of some had multiple whitish necrotic areas throughout the parenchyma. Mild oedema around the heart and lungs was also noted in some cases. One animal which was observed with respiratory distress subsequently died and was found to have the trachea almost totally occluded by a hyphal mass. Mycological culture of a cross-section of the portion of trachea affected yielded almost pure primary culture of *Aspergillus* sp.

Plate 7a: *C. porosus* hatchling showing necrotic areas along jaws and in buccal cavity (photo D. Steley).

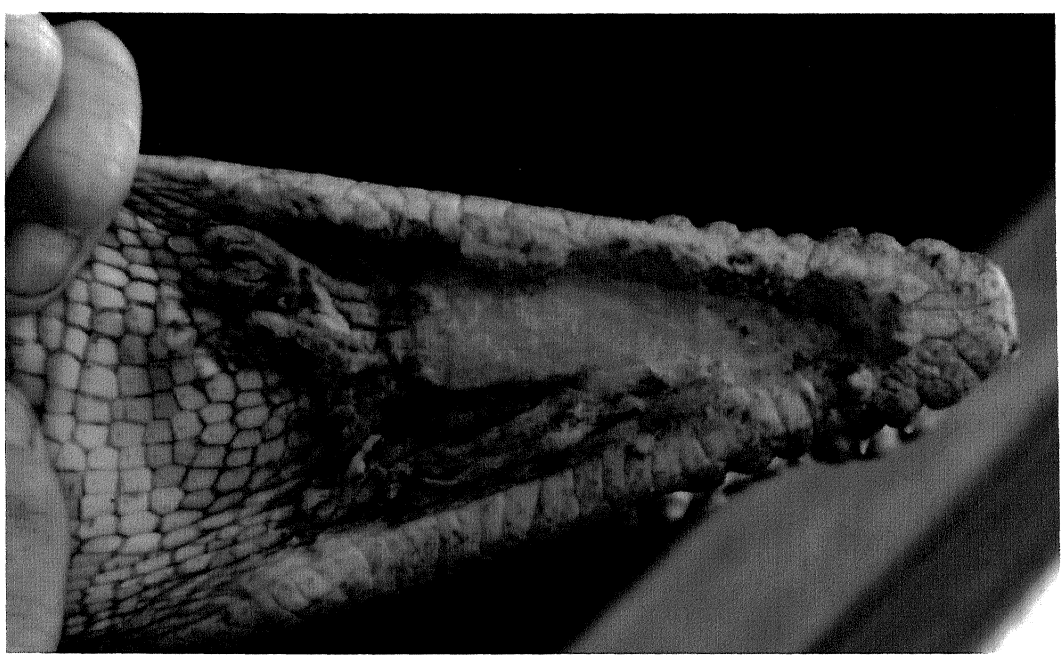

Plate 7b: Ventral surface of juvenile's jaw showing extensive fungal colonisation.

Plate 8a: Hind foot of *C. porosus* hatchling showing widespread necrotic lesions.

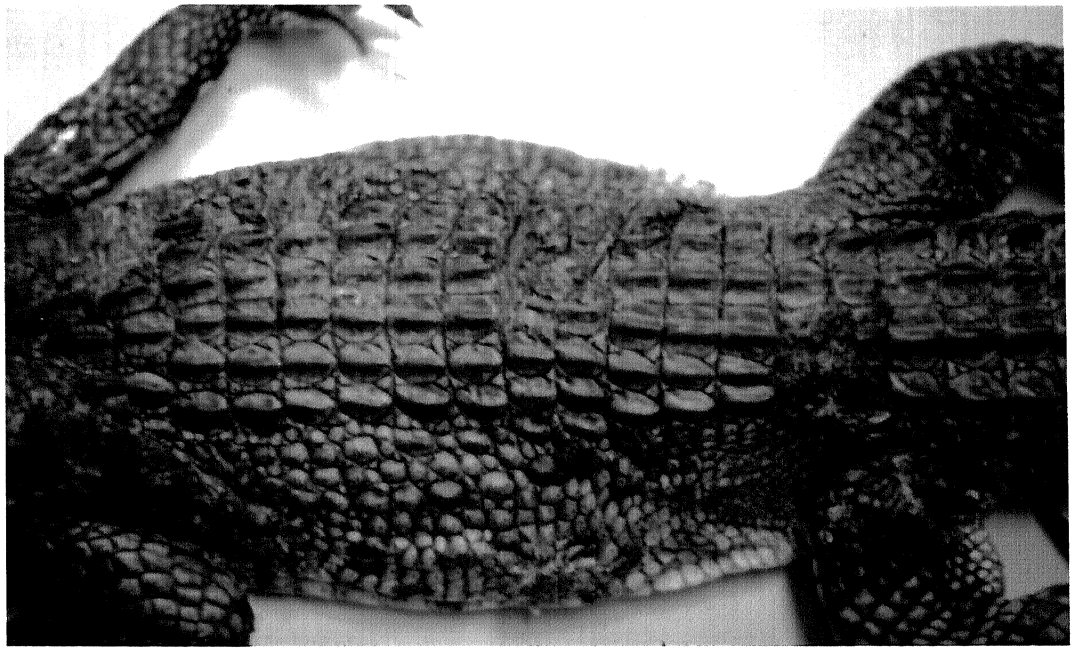

Plate 8b: *C. porosus* hatchling showing external lesions.

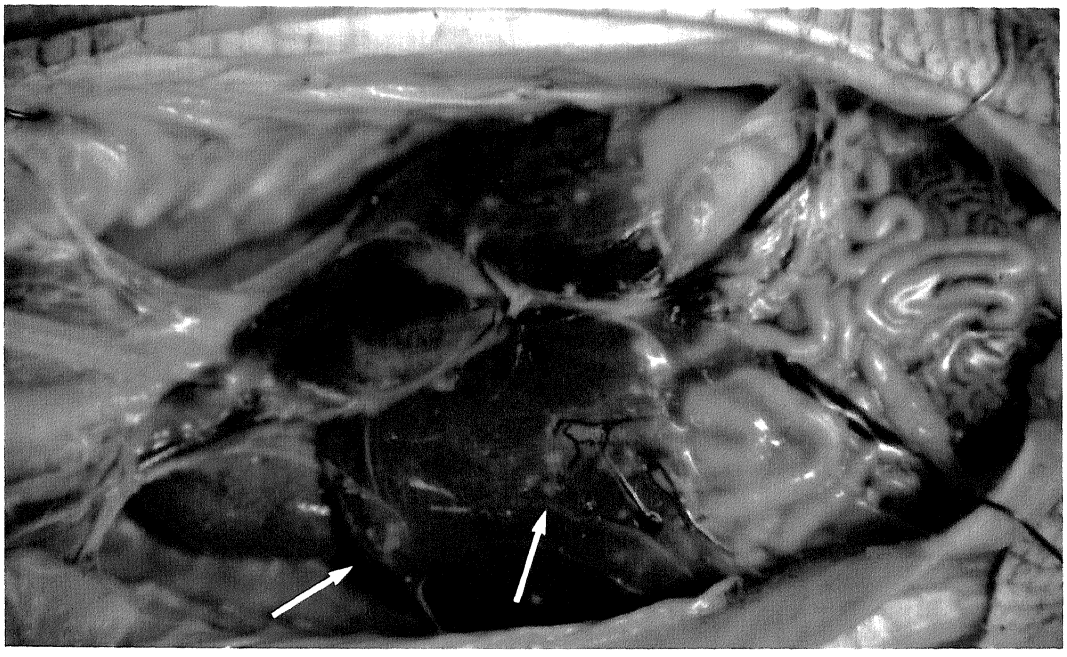

Plate 9a: Abdominal dissection of hatchling shown in Plate 8b showing hepatic lesions (arrowed).

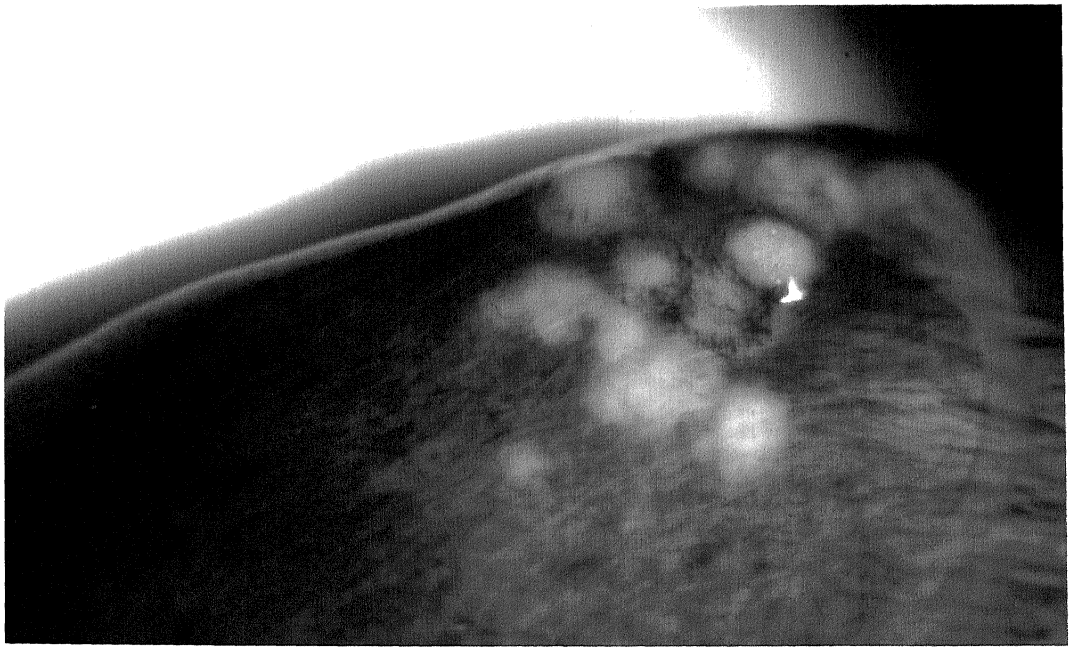

Plate 9b: Enlarged view of hepatic lesions in Plate 9a.

The majority of the diseased crocodiles were visibly affected externally. However on occasions animals which had no external symptoms were found dead and at post-mortem were observed to have moderate to severe internal infections. Those animals most often affected became lethargic and lost their appetite. This resulted in further loss of condition, general body weakness and poor muscle tone, with loose skin around the neck, along the sides of the belly and around the upper legs. Weight loss was common due to the disinclination to feed and a reduction or loss of the swallowing reflex. Of those that were force fed (including throat massage to stimulate swallowing), some continued to decline in condition, frequently regurgitating the food and remaining listless and anorexic. If force fed with the inclusion of vitamin supplements before too much body mass was lost, some animals recommenced feeding of their own accord. However, this only appeared to slow down their rate of decline and these animals later succumbed to the disease. As the animals became more ill there was a reduction in their normal behavioural reflexes. That is they lost their 'fight or flight' reflex when picked up and handled and were almost docile.

Those animals which were obviously diseased tended to position themselves in warmer parts of their pens. For the animals observed on campus this was most often under the infra red lamp on the basking rocks, or over the aquarium heater at the water inlet. In the pens at the farm this was in the area nearest the fan heaters, or when available, in the sunlit areas on the floor.

3.2 Histology

A frequent problem was post-mortem autolysis in the animals collected from the pens or presented for investigation. Although tissue from these animals was able to be sampled mycologically using the method of Harrower (1989a) it was not suitable for histological procedures due to decomposition.

Fungal hyphae were evident in mouth mucosa and gum, small intestine wall, liver, lung and epidermis. *C. porosus* liver section, stained by the PAS method and demonstrating branching septate hyphal elements, may be seen in Plate 10a. Mycologically stained microscope slide preparations made of wound exudates (smears or squashes) from various locations on the crocodiles' bodies were found to have fragments of hyphae present. Similarly, squashes or smears of tissue samples

taken from eggs showed evidence of hyphae. Gum scrapings stained by the PAS method and also showing the presence of hyphae may be seen in Plate 10b. Multiple fungal granulomas are evident in the section of *C. porosus* lung pictured in Plate 11a. An area of that lung section has been enlarged in Plate 11b and fungal hyphae are visible.

Results of histological investigations of autopsy tissues carried out at a Veterinary Pathology Laboratory at times of high hatchling and yearling morbidity and mortality also gave results similar to those presented in this thesis (Pierce and Hill, 1992, 1993). The presence of fungi was evident histologically in lung, liver, spleen, skin, and jaw.

3.3 Mycology

Surfaces tested, including walls and floors of incubation facilities and hatchling pens; freezer room shelves, walls and floor; feeding trays, animal food preparation equipment and work benches all proved positive to fungal contamination of varying degrees. Time of sampling before or after cleaning affected the degree of contamination. A variety of fungi were cultured, including *F. solani*, *Aspergillus* sp., *P. lilacinus* and *Cladosporium* sp. Cultures of *F. solani* derived from environmental sampling are shown in Plate 12a.

Soil and sand samples taken from various locations around the farm also proved positive to a similar wide variety of fungal species as did samples of natural nesting material. *F. solani* was readily isolated from nesting material sampled from one late nest collected in the latter part of the 1990/1991 breeding season. *F. solani* was also readily cultured from all nest sites sampled in the 1991/1992 breeding season.

Air settle plates from inside incubation facilities, in the work shed at the animal food preparation area, inside the hatchling building, and outdoors along thoroughfares between the work areas were all positive, with the degree of contamination directly in proportion to the time the plates were exposed. Settle plates set on windy days produced higher contamination levels. A variety of fungi were cultured, including *F. solani*. Samples of well water taken from immediately below the water surface, from the outlet at the top of the well, and from the outlet at the hatchling building were all positive for *F. solani*.

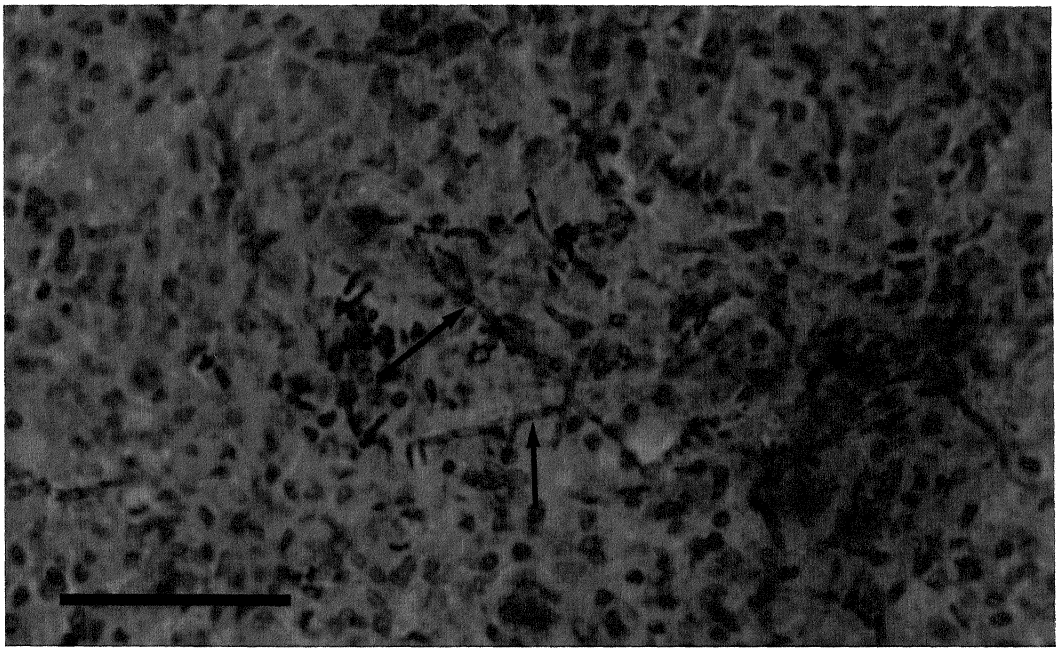

Plate 10a: *C. porosus* liver stained by PAS method, showing hyphal elements (arrowed). Bar = 50 μm.

Plate 10b: Gum scrapings from *C. porosus* hatchling showing hyphal elements (arrowed) stained by PAS method. Bar = 50 μm.

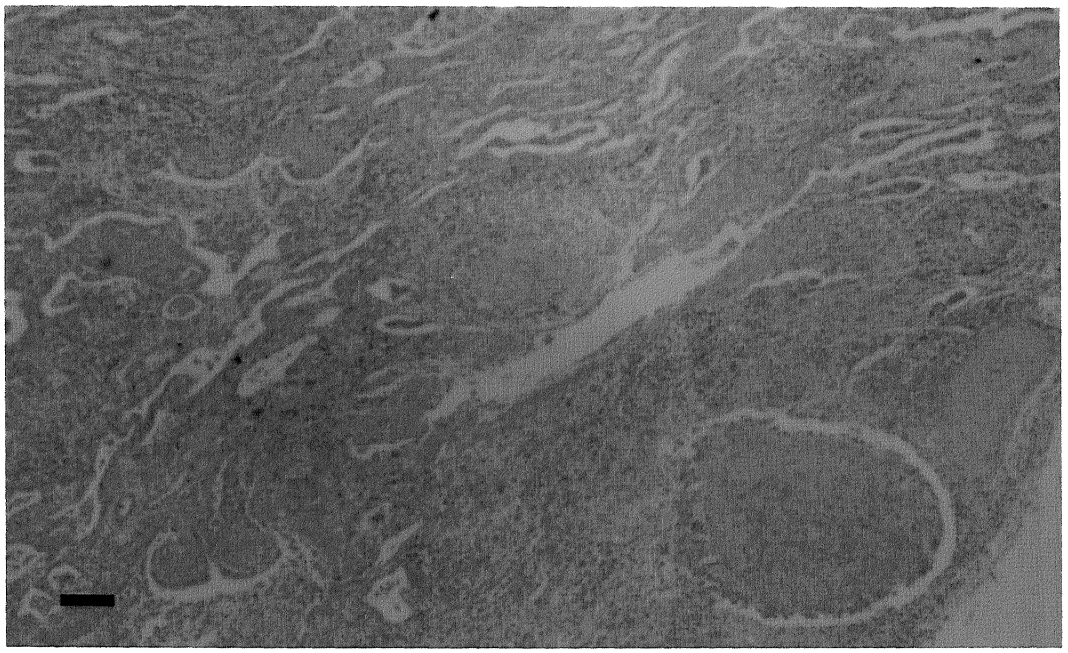

Plate 11a: *C. porosus* lung stained by the PAS method, showing multiple fungal granuloma. Bar = 100 μm.

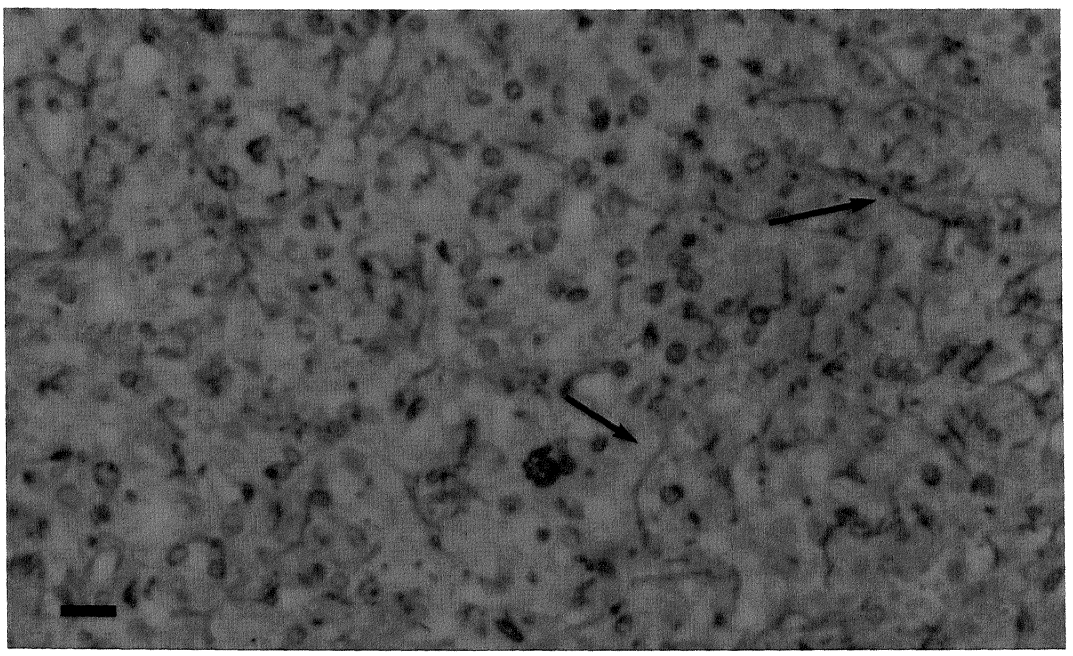

Plate 11b: Enlargement of Plate 11a, showing hyphal elements (arrowed) in the lung tissue, stained by the PAS method. Bar = 10 μm.

Sticky-tape samples taken from various surfaces in the incubation facilities before cleaning produced a range of fungi. Species isolated included *F. solani*, *Aspergillus* sp., *P. lilacinus* and *Cladosporium* sp. After surfaces had been cleaned with anti-fungal agents, samples taken after a one minute interval did not produce any fungal colonies. Low numbers of fungal colonies were cultured from samples taken after five minutes. Sampling at one hour after cleaning gave similar results to the samples taken before cleaning. Tape sections attached to microscope slides for immediate viewing showed a range of fungal conidia, pollen grains and organic debris.

Scrapings taken from various external skin lesions and around the teeth of the hatchlings produced an abundance of rapidly growing cultures of *F. solani* and some cultures of *Aspergillus* sp., and *P. lilacinus*. In some instances almost pure cultures were obtained from the original scraping. Post-mortem samples of crocodile tissue cultured using the method of Harrower (1989a) yielded primary fungal cultures with some bacterial contaminants. Purified mycelial cultures of *F. solani*, *Aspergillus* sp., and *P. lilacinus* were readily obtained. Frequently, almost pure cultures were obtained from the primary culture as shown in Plate 12b. Tissues or organs which yielded cultures of fungi were liver, lung, trachea, small intestine, skin from various body lesions, eyelids, and mouth mucosa. Results of mycological investigations of autopsy tissues carried out at a Veterinary Pathology Laboratory at times of high hatchling and yearling morbidity and mortality also gave results similar to those presented in this thesis (Pierce and Hill, 1992, 1993). Fungi were isolated from lung, liver, spleen, skin, and jaw. The predominant fungus was *Fusarium* sp., with *P. lilacinus*, *Mucor* sp., *Penicillium* sp., and *Aspergillus versicolor* also being isolated as well as some other unidentified species.

The oviducts from a breeding female, killed by another crocodile while defending her nest and later sampled and presented for mycological examination were positive to *Cladosporium* sp. However, this result may be spurious due to the method of collection and presentation of the tissue sample which had been taken after the skinning process.

In April and May of 1991 fungi were cultured from incubating eggs. Although *F. solani* was the predominant fungus, *P. lilacinus* and *Aspergillus* sp. were frequently isolated. From twenty-six clutches incubated in 1991, fungi were cultured from eggs

of eight clutches in the latter part of the season. Samples taken from the shells (refer Plate 13a) and shell membranes (Plate 13b) frequently gave positive fungal growth. Massive mycelial growth was also found in the air spaces of some eggs containing unhatched full term embryos (refer Plate 14a). In subsequent years, random mycological sampling from fertile eggs which ceased to develop also yielded fungal cultures (refer Plate 14b). Mycological samples taken from infertile eggs, the extra-embryonic membranes immediately after hatchling emergence from the egg (Plate 15a), early embryonic discs (Plate 15b), and from dead embryos also frequently gave positive fungal growth.

In the 1991/1992 breeding season each clutch of eggs was randomly sampled before washing, after washing, and after dipping. Results for unwashed eggs covered with their sticky mucous layer and adhering nesting material were similar to the results for nesting material. That is, there was an abundant quantity and variety of fungal species. After washing the eggs, with subsequent removal of contaminated nesting material, cultures from samples taken were greatly reduced in abundance. However, no single culture proved negative. Samples taken after dipping the eggs for five minutes in Arocide® made up at 1:150 were all negative for fungal growth. In subsequent breeding seasons, egg surfaces were only sampled mycologically after washing and after dipping. Results were similar. That is, low numbers of fungal colonies were isolated from washed surfaces and no fungal colonies isolated from dipped surfaces. Cleaned and dipped eggs which were 'speckled' or already cracked when initially placed in incubation were tested mycologically during subsequent incubation. Fungal cultures were isolated from the shell surfaces. These damaged eggs frequently ceased development. Cleaned, dipped and developing eggs without blemishes remained clear of fungal contamination throughout incubation.

3.3.1 Sensitivity Tests

Sensitivity tests by Harrower (1989b) indicated that *F. solani* was sensitive to Nitrofurazone. This substance is an anti-microbial frequently used in the aquaculture industry to combat fish diseases, and had been used successfully for some years at CQU Aquaculture Facility.

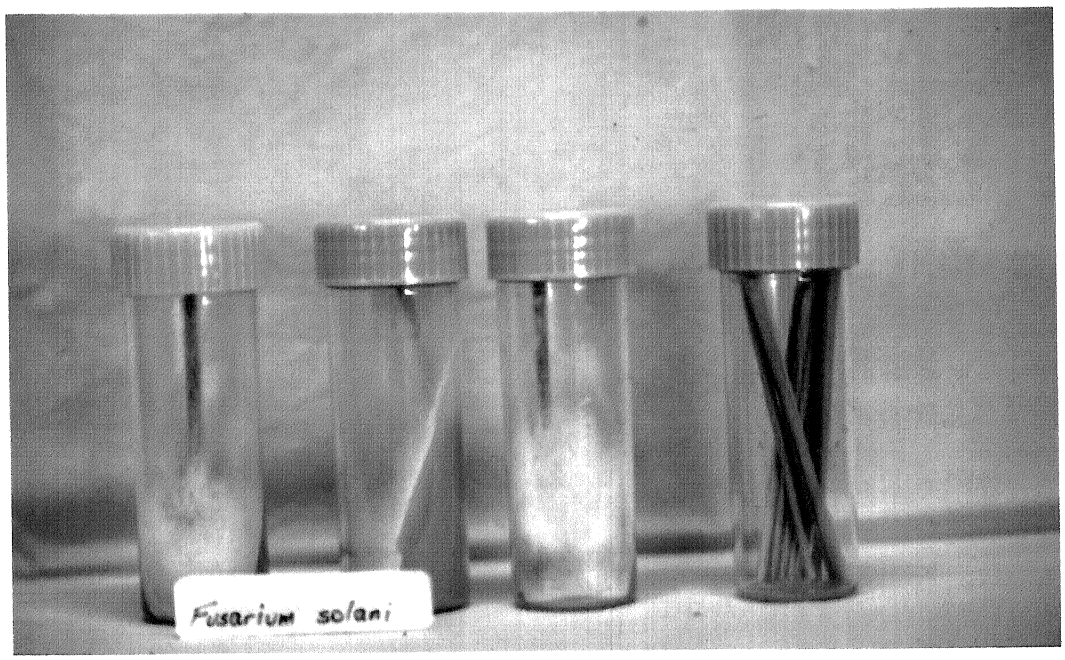

Plate 12a: Cultures of *F. solani* derived from environmental sampling.

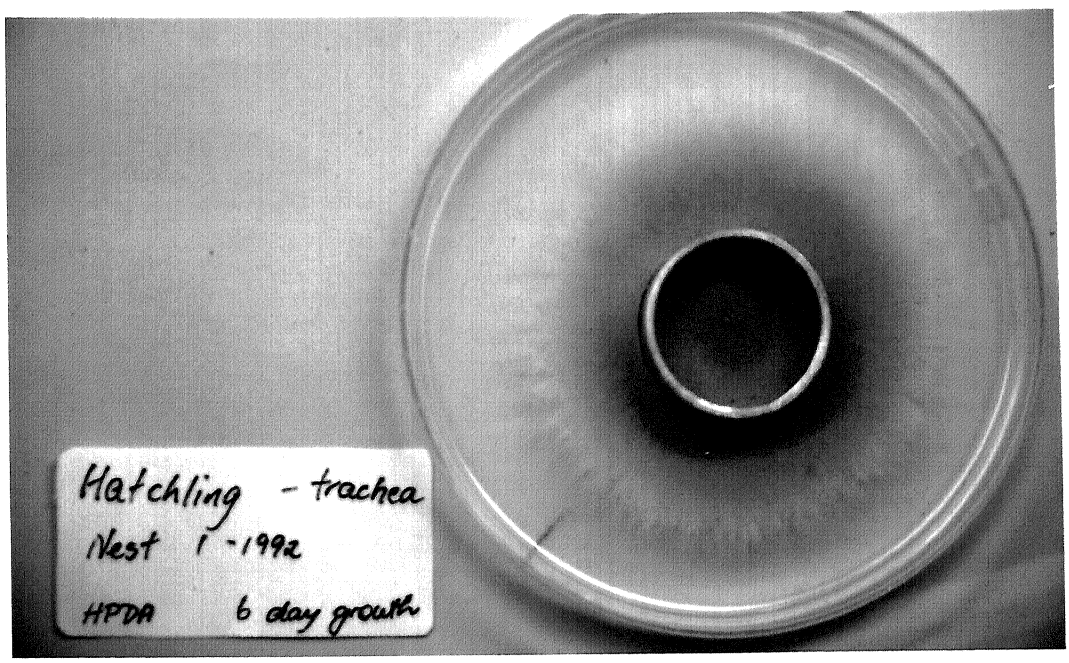

Plate 12b: Ring technique used for purifying mycelial pathogens of bacteria. Tissue fragments are placed within the ring.

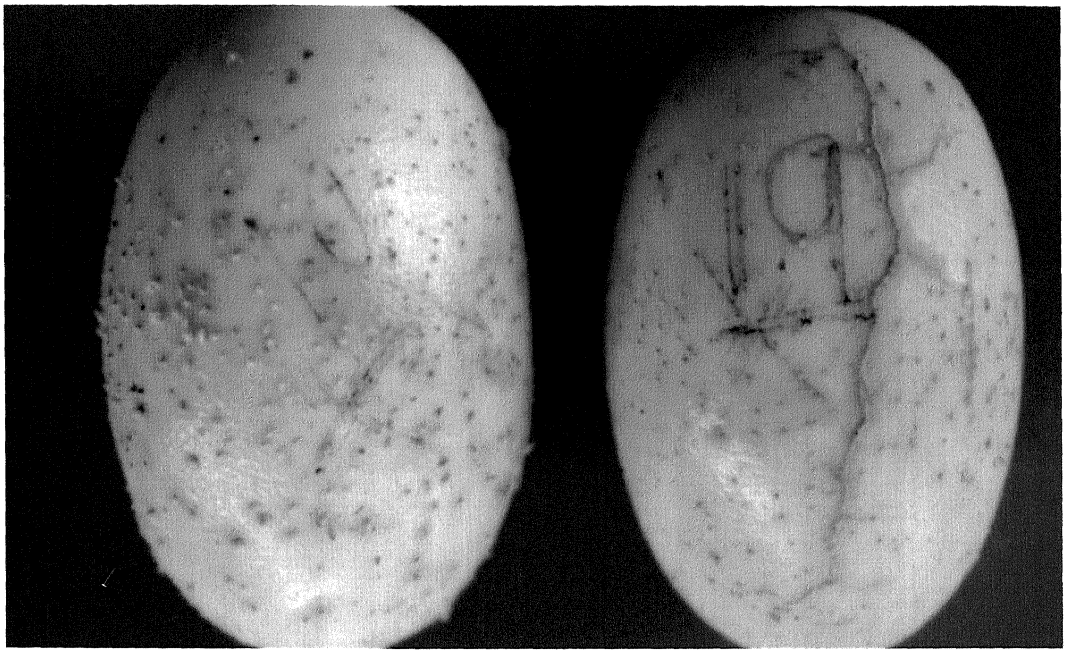

Plate 13a: *C. porosus* eggs with fungal colonies apparent on the surface.

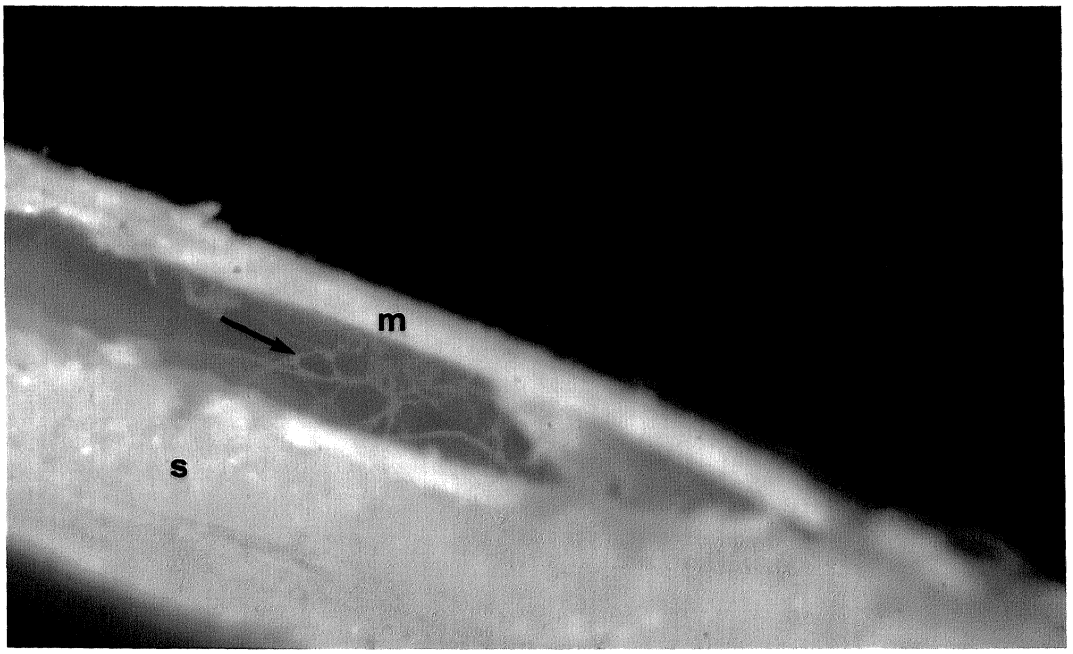

Plate 13b: Cross section of *C. porosus* egg shell (s) with membrane layer (m) attached. Hyphae (arrowed) can be seen in the air space between the two layers.

Plate 14a: Dead *C. porosus* embryo in egg, with fungal growth visible in air space (arrowed).

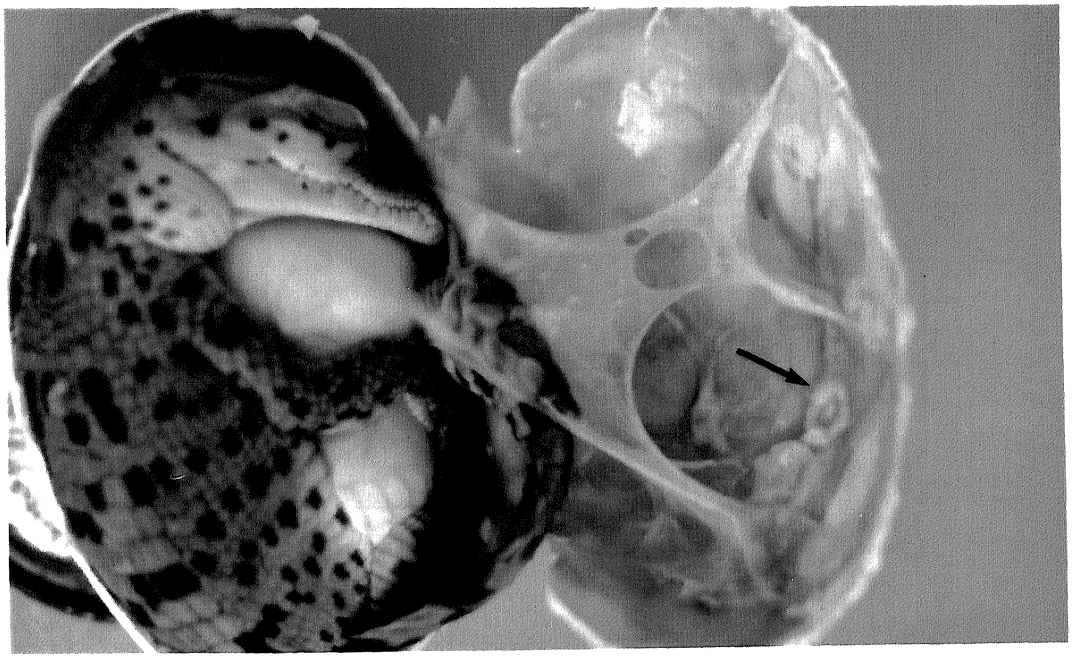

Plate 14b: *C. porosus* embryo at a later developmental stage. The fungal contamination (arrowed) has progressed deeper inside the egg through and from the membrane layer.

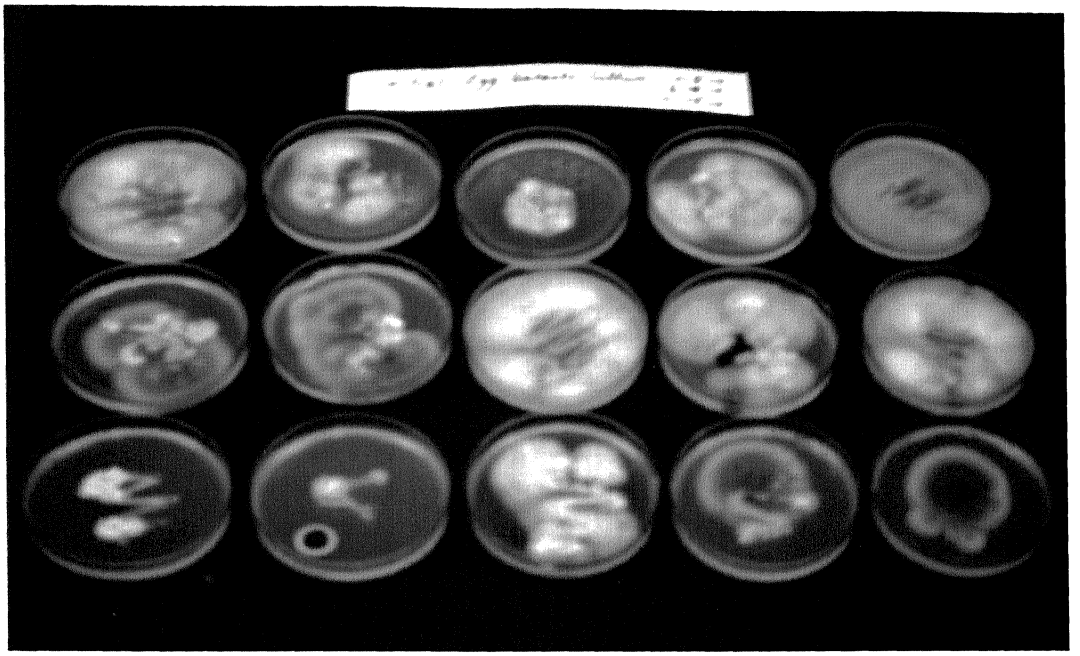

Plate 15a: Representative range of primary isolates obtained from egg tissue samples.

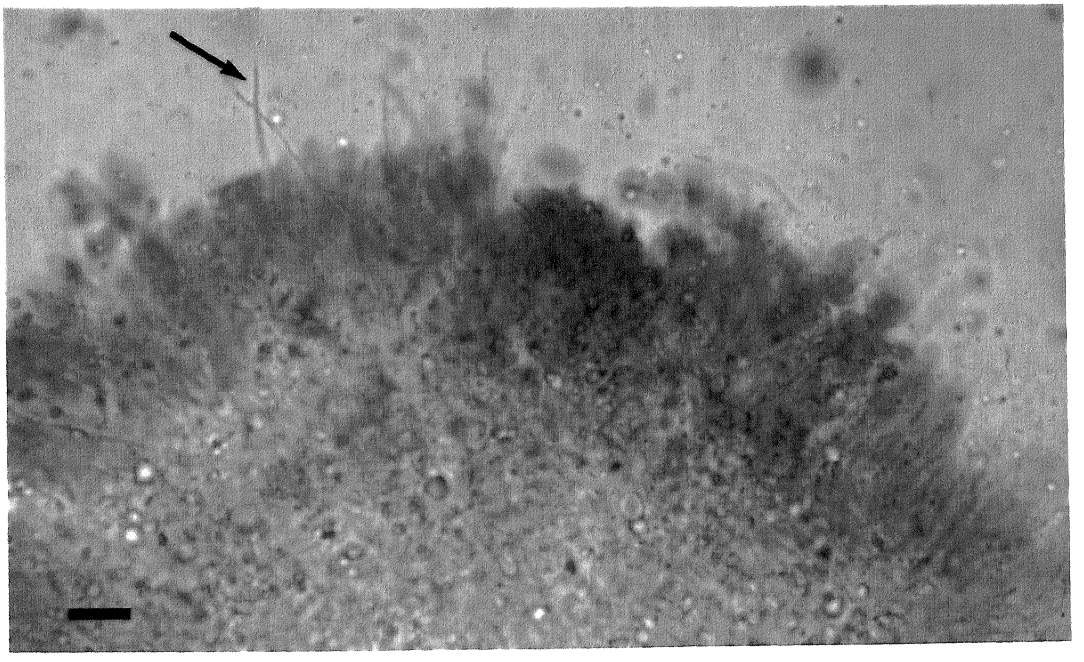

Plate 15b: Hyphal elements (arrowed) in infected tissue surrounding embryonic disc. Bar = 100 μm.

Based on the sensitivity tests and CQU experience, 10 mg of Nitrofurazone to each litre of water was used, with the preweighed powder added to the water in the hatchlings' pens after their cleaning and refilling. Treatment was predominantly to the hatchlings' external body only, with hatchlings swimming in or being immersed in the treated water. Small amounts of Nitrofurazone were ingested when the hatchlings drank the treated water. Results from a treatment trial using Nitrofurazone are detailed in section 3.11.1.

Sensitivity tests carried out on request by Lord (1990) showed *F. solani* to be particularly resistant to the common anti-fungal agents. The results are listed in Table 13.

Resistant	Sensitive
amphotericin B	econazole
ciclopirox	griseofulvin
clotrimazole	ketoconazole
flurocytocine	Tecto®
isoconazole	tioconazole
natamycin	
nystatin	

Table 13: Sensitivity Tests (Lord, 1990)

Of those agents to which *F. solani* was sensitive, Tecto® with the active ingredient thiabendazole was selected for trial. Usage was at the rate of 50 mg of thiabendazole powder per kilogram of hatchling body weight given at the first instance in suspension as a fluid drench and subsequently by addition to the food rations. Results from a treatment trial using thiabendazole are detailed in section 3.11.2.

Of the three products Arocide®, Virkon S® and Malachite Green which had been selected for testing for possible use as an egg disinfectant, only Arocide® proved effective against the four fungi which had been commonly isolated to that time. Results are summarised in Table 14.

	F. solani	*P. lilacinus*	*Aspergillus* sp.	*Cladosporium* sp.
Control No anti-fungal 1 minute	R	R	R	R
Control No anti-fungal 5 minutes	R	R	R	R
Arocide® 1:150 1 minute	S	S	R	S
Arocide® 1:150 5 minutes	S	S	S	S
Virkon S® 1:200 1 minute	S	R	R	S
Virkon S® 1:200 5 minutes	S	R	R	S
Malachite Green 5ppm 1 minute	R	R	R	R
Malachite Green 5ppm 5 minutes	R	R	R	R

Table 14: Sensitivity tests for egg treatments (R = resistant, S = sensitive).

Arocide® was subsequently used to disinfectant eggs. It was made up in tap water at a dilution of 1:150 and administered to cleaned eggs as a dip for five minutes, as outlined in sections 2.8.2, 2.8.3, and 2.8.4. Results for egg hatchability are detailed in section 3.8 and 3.12.

3.3.2 Characteristic Features of Commonly Isolated Fungi

Description was based on cultures grown on HPDA at 30°C and 37°C and identified with reference to Funder (1968); Booth (1971a); Toussoun and Nelson (1976); Koneman *et al* (1978); Nelson *et al* (1983); Barnett and Hunter (1987); Larone (1987); Carter and Chengappa (1991).

F. solani

Thermally monomorphic; surface white and fluffy; reverse non-pigmented; filamentous; growth rapid; septate hyphae; small oval microconidia 2-4 x 4-8 μm and large canoe shaped multi-septate macroconidia 3-8 x 11-70 μm produced from phialides on branched or unbranched conidiophores; macroconidia and conidiophores commonly seen as sporodochia resting on and interconnected by hyphal mass; chlamydospores formed in old cultures. *F. solani* hyphae, macroconidia and microconidia are shown in Plate 16a. Chlamydospores are shown in Plate 16b.

***Aspergillus* sp.**

Thermally monomorphic; colony surface at first white then green with velvety texture; reverse off-white to light gold; rapid growth;, septate hyphae; unbranched conidiophore which enlarged to form a vesicle covered with flask-shaped uniseriate phialides; conidia were spherical 2-5μm in diameter and basipetaly catenate. *Aspergillus* sp. conidiophores, phialides and conidia are shown in Plate 17a.

P. lilacinus

Thermally monomorphic; surface violet and powdery; reverse off-white; rapid growth; hyphae septate; resembled and may be mistaken for *Penicillium* sp. however the phialides of *Paecilomyces* were more elongated and tapered; elliptic or oblong conidia approximately 2 x 4μm occurring in long unbranched basipetal chains. *P. lilacinus* hyphae, tapered phialides and conidia are shown in Plate 17b.

***Cladosporium* sp.**

Thermally monomorphic; surface dark brown with velvety texture; reverse black; growth moderate; hyphae septate; conidiophores branched and produced conidia in two or more acropetal chains; small oval conidia approximately 3-6 x 4-12μm; detachment of the conidia left characteristic scars on the conidiophores or other conidia.

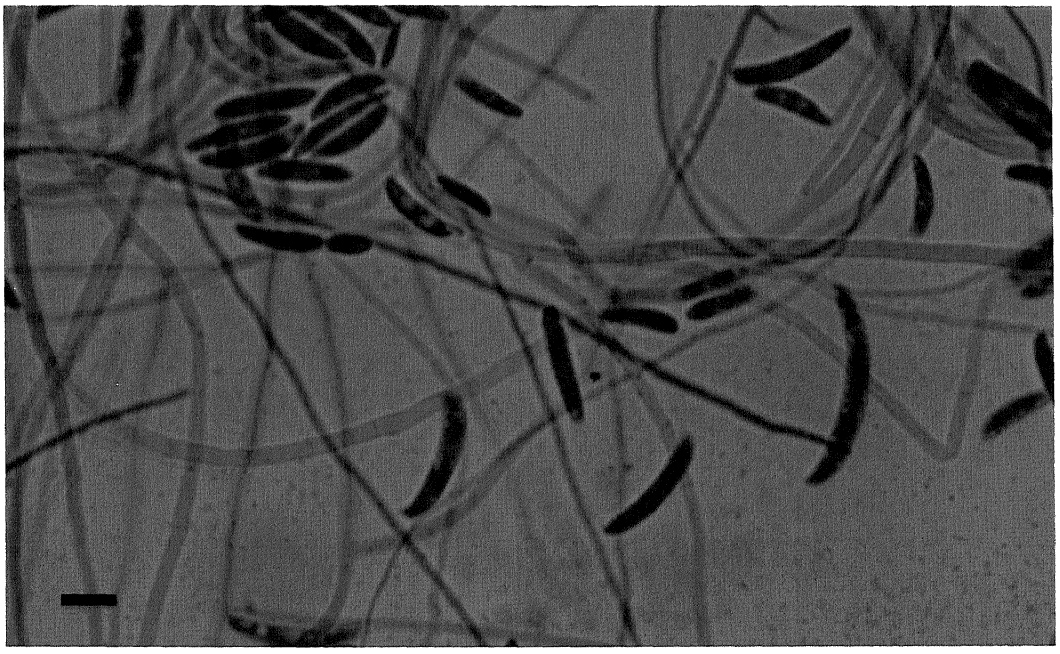

Plate 16a: *F. solani* hyphae, macroconidia and microconidia. Bar = 50 μm.

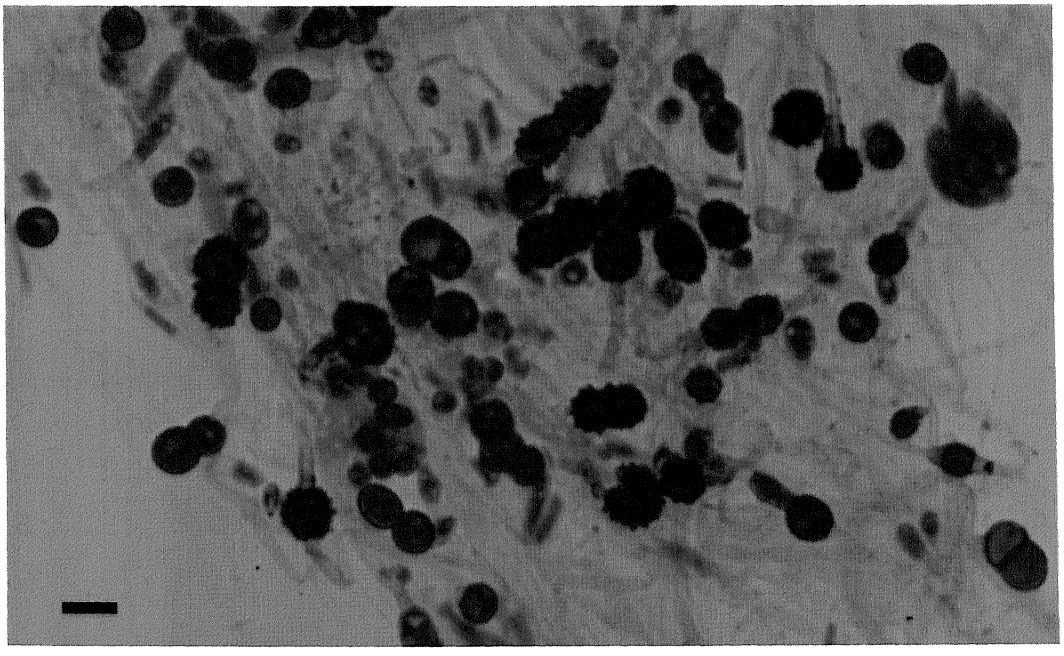

Plate 16b: Chlamydospores of *F. solani*. Bar = 10 μm.

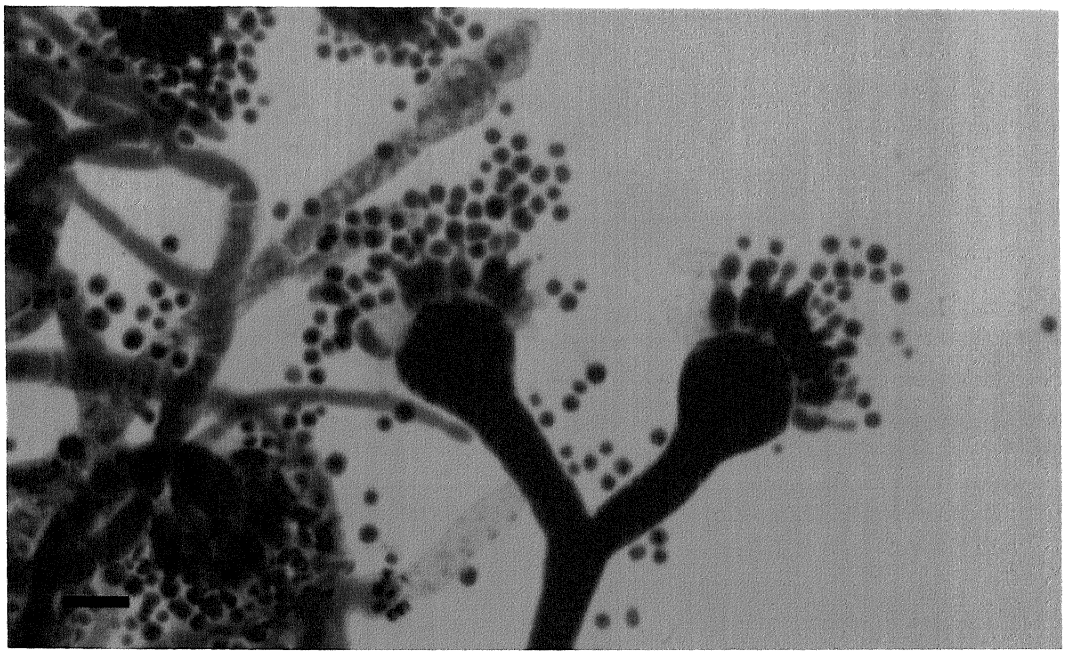

Plate 17a: *Aspergillus sp.* conidiophores, phialides, and conidia. Bar = 5 μm.

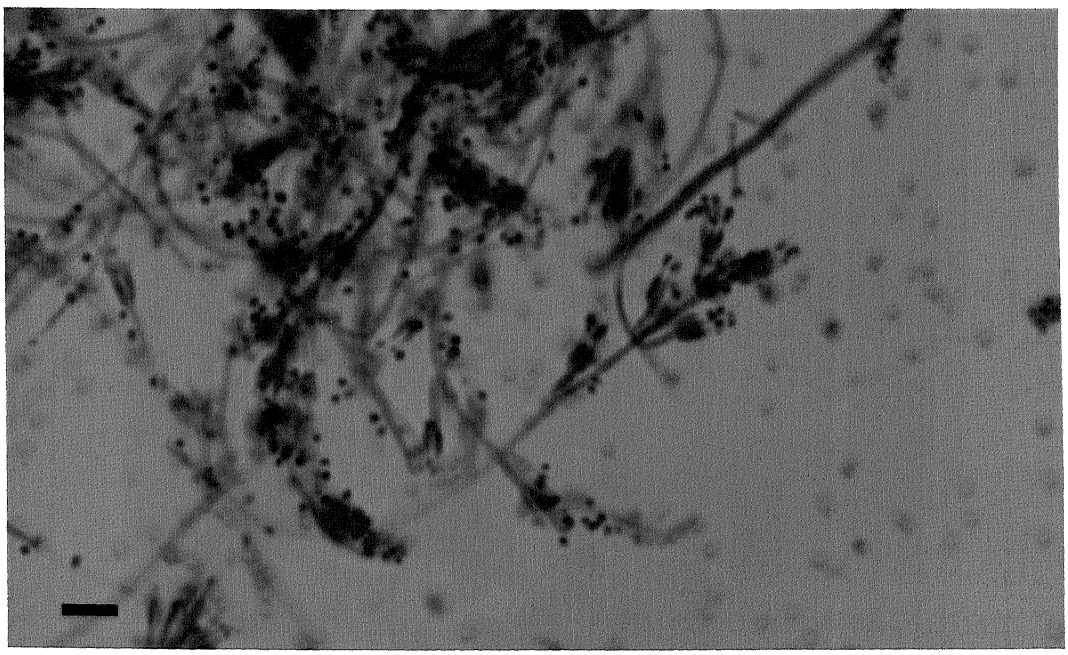

Plate 17b: *P. lilacinus* hyphae, tapered phialides, and conidia. Bar = 10 μm.

3.4 Haematology and Biochemistry

Haematology results are presented in Table 15. Abbreviations are described fully in Appendix 5. Pathology Laboratory results were obtained on the day of blood collection. Additional haemoglobin results were obtained on campus after twenty-four hours and after seventy-two hours.

Compared with the three healthy 3 year old crocodiles, lower erythrocyte counts, lower haematocrit values and lower haemoglobin levels were evident in the sick 12 month old crocodiles.

Age and health status	3 year old, healthy			12 month old, sick		
Sex	female		male	unknown		
Animal number	1	2	3	4	5	6
WBC (x 10^9/L)	+++	+++	+++	**	+++	+++
RBC (x10^{12}/L)	0.95	1.24	0.94	**	0.53	0.78
Hgb (g/L)	123	140	104	**	51	91
Hgb g/L (after 24 hr)	110	134	113	**	41	77
Hgb g/L (after 72 hr)	99	116	86	**	44	72
Hct (%)	23.4	30.9	23.2	**	11.7	17.7
MCV (fL)	246.6	249.0	246.5	**	220.6	226.8
MCH (pg)	+++	+++	+++	**	96.2	+++
MCHC (g/L)	525	453	449	**	436	514
RDW (%)	7.0	---	---	**	.0	27.2
PLT (x 10^9/L)	14	12	7	**	17	##
PCT (%)	0.007	0.006	0.003	**	0.012	##
MPV (fL)	5.2	5.3	4.8	**	6.8	##
PDW (%)	17.7	18.3	16.7	**	20.7	##

Table 15: Haematology of *C. porosus* (+++ = too high to register, --- = too low to register, ** = insufficient sample, ## = not quantifiable)

Biochemical serum profiles from both the Pathology Laboratory (A) (obtained on the day of blood collection) and Veterinary Pathology Laboratory (B) (submitted and tested after six days) are presented in Tables 16a and 16b. Abbreviations are described fully in Appendix 5.

Age and health status	3 year old, healthy						12 month old, sick					
Sex	female				male		unknown					
Animal number	1		2		3		4		5		6	
Laboratory	A	B	A	B	A	B	A	B	A	B	A	B
Total protein (g/L)	66	68	65	68	61	64	59	60	30	29	64	64
Albumin (g/L)	<1.0	23	<1.0	22	<1.0	22	<1.0	17	<1.0	10	<1.0	18
Globulin (g/L)		45		46		42		43		19		46
A/G ratio		0.51		0.48		0.52		0.40		0.53		0.39
Total bilirubin (µmol/L)	18	14	17	3	18	5	9	4	7	1	13	5
Creatinine (µmol/L)	57	50	48	42	57	47	34	33	46	34	33	39
Urea (mmol/L)	6.9	7.5	5.5	6.0	1.7	1.7	0.9	1.1	1.0	1.1	1.0	1.3
CPK or CK (IU/L)	2981	3525	1099	1185	2021	2370	561	949	488	543	>4100	3735
GGT (IU/L)	1		2		1		3		1		2	10
AST (IU/L)	90	86	84	81	117	105	53	64	47	43	48	71
GLDH (IU/L)		8		10		20		13		13		8
Alkaline phosphatase (IU/L)	156		81		82		59		21		66	
Amylase (U/L)	86		40		38		28		14		**	
ALT (IU/L)	24		42		<5		19		11		**	
LDH(IU/L)	626		609		**		**		**		**	

Table 16a: Serum profiles of *C. porosus*. (** = insufficient sample, shaded boxes = tests not performed)

Age and health status	3 year old, healthy						12 month old, sick					
Sex	female				male		unknown					
Animal number	1		2		3		4		5		6	
Laboratory	A	B	A	B	A	B	A	B	A	B	A	B
Uric Acid (mmol/L)	>0.71		>0.71		>0.71		0.50		0.44		**	
Calcium (mmol/L)	3.27	3.42	3.32	3.57	3.31	3.57	2.54	2.85	2.38	2.49	2.81	3.10
Sodium (mmol/L)	172		167		165		153		145		151	
Potassium (mmol/L)	6.6		5.8		5.5		5.7		5.1		5.3	
Chloride (mmol/L)	113		119		93		114		111		118	
CO_2 (mmol/L)	7		7		14		11		15		13	
Glucose (mmol/L)	9.5		8.7		7.8		3.0		4.1		7.3	
Magnesium (mmol/L)	1.5	1.68	1.46	1.6	1.35	1.5	1.03	1.2	0.85	0.89	1.16	1.31
Phosphate (mmol/L)	>3.9		>3.9		3.16		0.85		0.78		1.08	
Copper (µg/L)		750		815		865		**		355		**
Zinc (µg/L)		790		1175		1590		**		565		**
Anion Gap	59		47		63		34		24		25	
Osmolality	345		334		325		297		284		298	

Table 16b: Serum profiles of *C. porosus*. (** = insufficient sample, shaded boxes = tests not performed)

Compared with Crocodiles 1, 2 and 3, crocodiles 4, 5 and 6 recorded lower levels of bilirubin, urea, amylase and uric acid. Total protein and albumin levels were lowest in animal 5, as was alkaline phosphatase. CPK was highest in animals 1 and 6. Calcium, glucose, magnesium levels were lowest in animals 4, 5 and 6, as were anion gap and osmolality values.

3.5 Hypoglycaemia

Haematology results from the Veterinary Pathology Laboratory for animals 1 and 3 are presented in Table 17. Plasma biochemistry results for animals 1, 2 and 3 are presented in Table 18 with abbreviations for both figures described in Appendix 5. Insufficient blood was obtained from animals 4 and 5 for analysis. The blood sample for the haematological profile from animal 2 was not suitable for testing as was the initial sample from animal 1. The second set of samples collected from animal 1 for both haematological and biochemical profiles were taken after the animal had already been dosed with glucose therefore the plasma biochemistry would have changed from the pre-treatment levels. This is reflected in the plasma biochemistry results in Table 18 showing similar glucose levels for animals 1, 2 and 3. CPK is high in animal 1.

Age and sex	18 months old, male	
Health status	Suspected hypoglycaemic	Healthy
Treatment	After glucose treatment	No treatment
Animal number	1	3
WBC (x 10^9/L)	14.0	17.0
RBC (x10^{12}/L)	0.96	0.98
Hgb (g/L)	83	82
PCV (%)	24	23
MCV (fL)	250	235
MCH (pg)	86.5	83.7
MCHC (g/L)	346	357
Neutrophils (%)	30	8
Lymphocytes (%)	54	77
Monocytes (%)	2	3
Eosinophils (%)	3	1
Other Cells (%)	11	11

Table 17: Haematology of *C. porosus* (suspected hypoglycaemic)

Age and sex	18 months old, male		
Health status	Suspected hypoglycaemic	Healthy	
Treatment	After glucose treatment	No treatment	
Animal number	1	2	3
Total protein (g/L)	50	47	50
Albumin (g/L)	25	24	24
Globulin (g/L)	25	23	26
A/G ratio	1	1.04	0.92
Total bilirubin (μmol/L)	1	1	1
Creatinine (μmol/L)	29	19	21
Urea (mmol/L)	0.6	0.6	0.6
CPK or CK (IU/L)	8325	4620	717
GGT (IU/L)	5	5	1
AST (IU/L)	76	89	47
GLDH (IU/L)	19	28	33
Calcium (mmol/L)	**	**	**
Glucose (mmol/L)	5.3	5.4	5.2
Magnesium (mmol/L)	0.89	0.81	0.81

Table 18: Plasma profiles of *C. porosus* (suspected hypoglycaemic) (** sample not suitable)

3.6 Environmental Parameters

3.6.1 Temperature

At the start of the research program air temperature in the A-frame, according to maximum/minimum thermometers, ranged between 25°C and 35°C but did not give any indication as to when or how long temperatures were at these extremes. Introduced usage of the Hisamatsu thermohygrograph with weekly re-positioning within the A-frame showed that air temperature minima were recorded between the hours of 1 am and 6 am, with the coldest usually just before dawn. Air temperature maxima were recorded between 12 noon and 3 pm. Warmer or cooler areas within the A-frame were determined after weekly relocation of the instrument. This enabled re-

positioning of the heating (thermostat controlled heater fans and oil heater) and cooling devices (electric fans) to eliminate such variations. Night time use of the fan heaters even during summer months was increased to raise the minimum air temperature to around 30°C. Natural ventilation was also increased during the hottest part of the day to reduce the maximum to 34°C. Use of the data logger further fine tuned air temperature control.

Similarly air temperature recording by maximum/minimum thermometers, Ota thermohygrograph and data logger in the egg incubation room enabled better temperature regulation and the prevention or elimination of hot or cold spots. The temperature within an egg housed in damp vermiculite was found to be 0.5C° below that of the vermiculite, which was 0.5 to 1C° cooler than the room air temperature. This temperature buffering effect of the vermiculite helped prevent egg cooling when new clutches were being added to the incubator room, or overheating when the thermostat controlled heater fans turned on. Temperature dependent alarms were activated initially at 27°C minimum and 35°C maximum. However, the temperature range was later reduced to 29°C minimum and 34°C maximum. Temperature controls for the incubation room were adjusted to 33°C to enable an egg temperature of 32°C.

Water temperature in the A-frame was not initially measured. However, after the availability of the data logger this was possible. It was found that in autumn, winter and early spring, the temperature of the tap water when it was introduced to the hatchling pens was below 25°C, and frequently in the range 15°C to 20°C. In mid winter this reduced further to from 10°C to 15°C, and even in mid-summer was below 25°C. When water from the pens was flushed out, it was in the temperature range of 30°C to 32°C. Therefore, temperature shock to the hatchlings would occur when the much cooler water was used to clean and refill the pens. Through data logger use, it was also found that the newly introduced cold water in the pens took up to six hours to reach a temperature similar to that of the ambient air temperature. The water supply was subsequently re-routed into a concrete holding tank fitted with a temperature-adjustable gas hot water system at the water outlet. Temperature was set at 35°C to allow for cooling through the pipes between the storage tank and the A-frame and incubation room.

3.6.2 Humidity

Initially humidity in the A-frame or incubation room was measured using a whirling hygrometer and a wet and dry bulb thermometer. However, these were of little value since both gave only the current humidity level. Introduced usage of the Hisamatsu thermohygrograph showed humidity levels of around 75 to 85 % dependent on the A-frame internal temperature and external weather conditions. Even with the A-frame external door open to allow for daily cleaning or to facilitate additional cooling, humidity was still maintained above 60%.

Humidity measurement in the incubation room was more critical and the initial use of the Ota thermohygrograph indicated humidity levels in the range of 80 to 100%. The more accurate data logger humidity probe determined room humidity levels of 95 to 99% with drops to 85 to 95% when the door was left open during the addition of additional clutches to the incubation trays (the longer the door was open, the greater the humidity drop). However, humidity levels within the damp vermiculite, similar in position to incubating eggs, were consistently 99 to 100%, even during access to the room by staff.

3.6.3 Light Regimes

No light intensity measurements were recorded. The hatchlings in the A-frame only had access to natural transmitted light through translucent skylights, or in some pens for short periods during the day, to sunlight. The upper pens on the left were in darkness except for any natural reflected light when their doors were open to enable cleaning or feeding. No behavioural differences were noted between hatchlings in the light or dark pens. Results for trials comparing growth in darkness and light may be referred to in section 3.11.5. The incubation room was in total darkness except for when staff were working there.

3.7 Husbandry

3.7.1 Housing

Nally tubs were suitable for housing only the smallest of hatchlings as the animals quickly learnt to pile in the corners and escape over the sides. The addition of lids

with observation windows prevented further escapes. Purpose-built free-standing tanks were the main location for housing hatchlings of increased size. However, some of the smaller active hatchlings were able to escape through the mesh lids prior to the temporary addition of styrofoam covers which were later permanently replaced by fitted shadecloth.

The first hot-room was too small and difficult to clean and was replaced by the larger incubation room. This easily maintained facility was used for egg incubation during the breeding season, and could be quickly modified for housing hatchlings from hatching until graduation to the A-frame immediately prior to the next breeding season. The A-frame was accessible and easily maintained but by the completion of this research was becoming over-crowded due to the incorporation of additional pens necessary to accommodate the rising numbers of surviving hatchlings.

3.7.2 Cleaning

The use of Glochlor bleach was generally successful in the daily cleaning of pens, food trays and food preparation implements. In areas of built up grime or where algae appeared in the pens, the use of Arocide® at recommended rates was successful. Arocide® was also used extensively for disinfecting 'sick' bays, or before transferring animals to previously used pens, as well as for preparing the incubation room at the start of the breeding season. The working solution was easily prepared and was fast and efficient in removing mould, algal growths, or other organic deposits. Arocide® was also found to be efficacious in the disinfection and cleaning of eggs for incubation (refer section 3.8).

The use of Tricon® as a preventative measure against bacterial infection of the incompletely closed abdominal wall of some newly hatched crocodiles appeared to be successful, with continued development and growth of those hatchlings so affected.

Aroclenz® was found to be particularly useful in the cleaning and healing process of fight wounds and other skin lesions affecting juvenile crocodiles. Its ease of usage by topical spray application without the need to handle the animals allowed for less stressful wound treatment, both to the affected animals and to the staff involved. The dark violet colour of the product also enabled easy visual identification of treated animals. Wounds treated with Aroclenz® healed faster than similar untreated wounds.

The use of this product was expanded to the treatment of wounds affecting sub-adult or adult crocodiles recuperating from territorial fights, with similar beneficial effects.

3.7.3 Food And Feeding

Calcium di-phosphate and iodised salt were the standard basic food supplements for all juvenile crocodiles. A vitamin premix, Feramo D®, included in previous years and prior to this study had been discontinued due to supply problems. The addition in late 1990 of a locally sourced vitamin and mineral supplement, Vita-Stress® was found to be beneficial to growth of juvenile crocodiles To again eliminate supply problems, Pet-Vite® was later substituted as the regular supplement and similarly proved beneficial to crocodile growth. In a growth trial Enzactiv® (refer section 3.11.3) was also added to the meat in combination with the other supplements.

Changes in meat type fed to the juvenile crocodiles at Koorana Crocodile Farm caused reductions in consumption for up to a week (Reimers, pers. comm.). In times of shortages, horse and predominantly beef were allocated to the hatchlings and yearlings rather than to older crocodiles. Horse meat was the preferred diet of the juveniles and was consumed in the highest quantities when available, followed by beef. Pig, poultry and kangaroo was the least preferred by the juvenile crocodiles, and in addition left a fatty scum in the water and on the pen floors, creating additional cleaning requirements.

3.7.4 Heating and Cooling

Heating by the use of the Maxigrow Turbo Aire 2000W heater fans in the incubation room and in the A-frame was effective as assessed by reference to data-logger data. The free standing oil heater although effective, was bulky and could not be placed in the pens as the crocodiles could sustain burns unless prevented from contact. Its location in the central corridor reduced access by staff. Cooling methods by electric fans and natural ventilation, although basic were effective when necessary.

3.7.5 Personnel

Restricted access by unauthorised staff or visitors to incubation facilities and hatchling and rearing pens was in force as far as possible. When non-regular or

substitute staff were required to enter those areas, the crocodiles became visibly agitated and 'piled' in corners or under the 'hides' with the increased possibility of their suffocation or other physical damage. If under prolonged stress (*eg.* due to presence of construction staff) the animals also became more difficult to handle.

3.8 Egg Incubation

Overall yearly egg and hatchling production from 1984 through to the cessation of this study may be referred to in section 3.12. Results for the years 1984 to 1991 inclusive were provided by Koorana Crocodile Farm.

3.8.1 1990/1991 and Previous Breeding Seasons

Prior to this study, egg losses during incubation were in excess of 50% (Lever, pers. comm.). The purpose-built shelved incubator with open racking used in the 1990/1991 breeding season was not entirely successful with hot spots in some sections and variability in humidity. Any existing or introduced contaminants were readily distributed throughout the incubator by the fan heater. Because of the open racking system the incubating eggs were subjected to contamination, and temperature and humidity fluctuations.

Mycological sampling of some eggs which ceased to develop from eight clutches late in the incubation process resulted in fungal cultures, predominantly *F. solani*. Hyphae were visible in the air spaces of some unhatched full term eggs. Samples of nesting material from the last nest of the season yielded a range of fungi, including *F. solani*.

3.8.2 1991/1992 Breeding Season

A breeding female's oviducts which were presented for mycological examination proved positive to *Cladosporium* sp. However, the tissue sample had been taken at the farm after the skinning process (refer section 3.3) and results may, therefore, be meaningless.

Mycological cultures from all nests which were all sampled during this breeding season produced a wide range of fungal species in large numbers. *F. solani* was cultured from each isolate thus showing it to be widespread over the farm environment.

The use of Arocide® as a dip at a dilution of 1:150 for 5 minutes was successful in eliminating contamination from the surfaces of the eggs. Mycological cultures taken from egg surfaces before washing produced large numbers and varieties of fungal species. Samples taken after washing produced cultures fewer in number and less variety. No samples, however, gave nil growth. Samples taken from the egg surface after the Arocide® dip were all negative. During incubation, some eggs which had initially tested negative after dipping, subsequently ceased development and were found to have fungi growing on the shell surface and internally. These were eggs which were either cracked, dented, or still appeared 'speckled' after dipping.

Although ambient room temperature and humidity were acceptable according to instrumentation data, the use of dry vermiculite appeared detrimental to egg incubation with some eggs dehydrating and ceasing development. These eggs were found to have very large air spaces between the membrane and shell and were negative to fungal culture.

A cyclone which affected the district on the night of 15 March 1992 caused a power cut to the farm and incubation facilities. Generators were used as an interim back-up power supply. The subsequent power surge when mains power was restored caused a malfunction in the incubation room heaters so that they did not turn off at the pre-determined maximum temperature. Instrumentation data during this period revealed over-heating in the upper trays of eggs which correlated with cessation of development of eggs from those trays. As results from mycological tests were negative, those egg losses were attributed to over-heating directly related to the effects of the passing cyclone.

High embryonic deaths (refer section 3.12) for the 1991/1992 season can be attributed to dehydration and over-heating as well as to fungal infection.

3.8.3 1992/1993 Breeding Season

Mycological testing of the surfaces of eggs after washing and after dipping were similar to the previous season. That is, few species in low numbers were cultured after washing, and negative cultures after the Arocide® dip. As in the previous season, eggs which were either cracked, dented, or still appeared 'speckled' after dipping later developed fungal cultures both on the surface and internally.

The use of moist vermiculite created a temperature buffering effect on the eggs which prevented their over-heating or cooling during or after power cuts. Temperature monitoring by various methods indicated the desired temperature range was being achieved and maintained (refer section 3.6.1). The vermiculite, sufficiently moist that droplets of water could be squeezed out, as used by Zimbabwean and South African farmers (Blake, 1991), was too moist for the humid conditions in the incubator room. Eggs from early nests absorbed moisture and swelled to the point of cracking the shells but not actually splitting the inner membranes. These eggs were carefully relocated to new trays containing vermiculite which was damp but not as moist as previous. Under the new conditions the majority of the swollen eggs shrank to normal size and continued normal development. Subsequent clutches of eggs incubated in damp vermiculite (but not too moist) also developed normally. Humidity levels within the damp vermiculite, as recorded by the data-logger, were consistently high (refer section 3.6.2).

3.8.4 1993/1994 Breeding Season

During this season all eggs were cleaned using warm water approximately 3C° higher than that of the recorded nest temperature at egg collection. Dipping the cleaned eggs in Arocide® at the same temperature as the water wash was then carried out using the same procedure as the previous year. Embryo deaths still occurred but in greatly reduced numbers (refer section 3.12). Damaged or 'speckled' eggs were predominantly those which ceased development and subsequently produced fungal cultures. Late clutches during this breeding season produced a higher proportion than normal of infertile eggs as well as fertile but damaged eggs.

Regulation and monitoring of temperature and humidity in the incubation room had been modified and refined, with additional alarms and back-up systems, in order to reduce or eliminate further problems.

3.9 Photography

Colour photographs are presented as Plates 1 to 17b in this thesis. Black and white prints are presented as Plates 18a to 20b. Unless otherwise acknowledged all photographs were taken by the author.

3.10 Scanning Electron Microscopy

The shell pore size of *C. porosus* eggs varied from 100 μm to 500 μm (refer Plate 18a). Pores were frequently plugged with hyphal elements and organic debris.

Fungal colonies also proliferated along cracks in the shell. Conidia and conidiophores of different fungal species were visible on the shell surface and in open pores (refer Plates 18b, 19a). These fungal elements were similar in shape and size to those of *Aspergillus* sp. (refer Plate 19b), *Cladosporium* sp., and *F. solani* (refer Plate 18b). Hyphae were visible penetrating between the shell calcite layers (refer Plate 20a). An unidentified species of mite with body width of less than 100 μm was observed on the internal surface of some shells (refer Plate 20b).

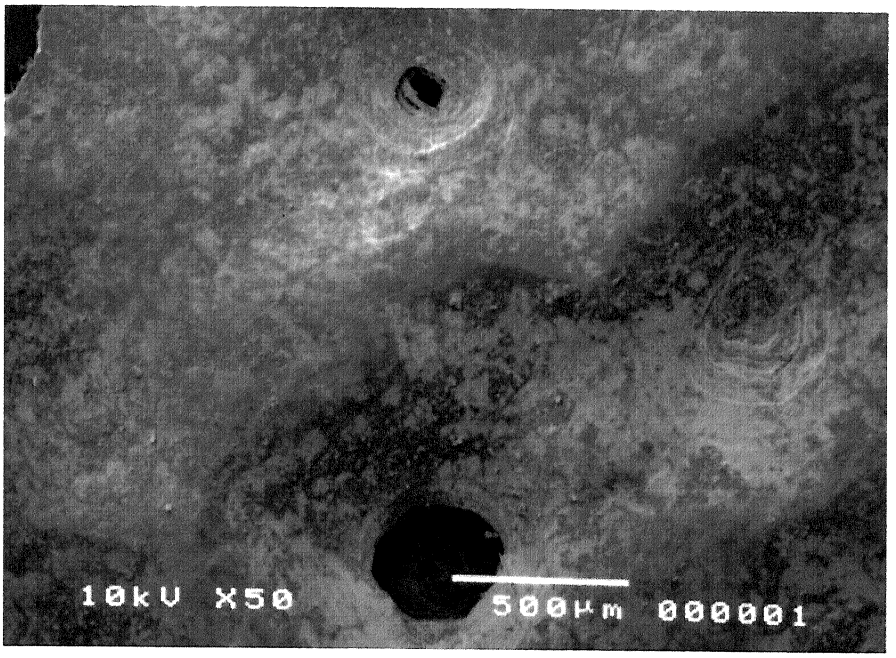

Plate 18a: External surface of egg shell of *C. porosus* showing pore structure. Bar = 500 µm.

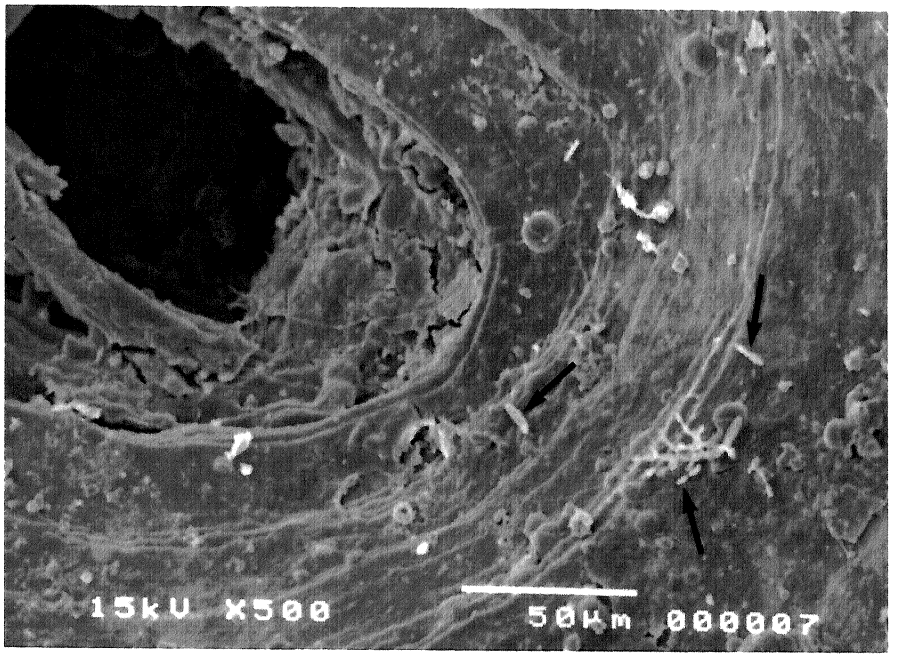

Plate 18b: Enlargement of Plate 18a, upper pore, showing conidia (arrowed). Bar = 50 µm.

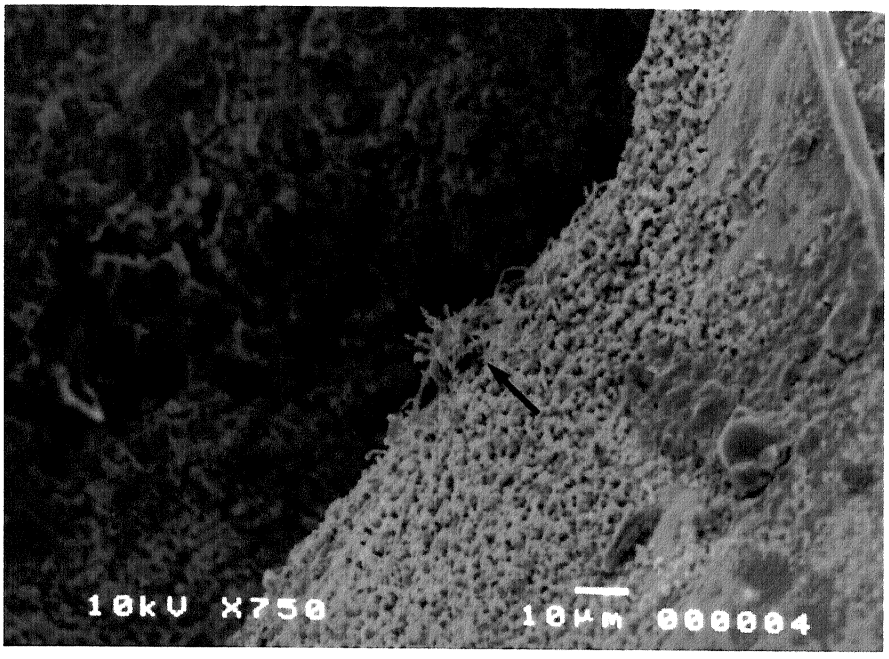

Plate 19a: Enlargement of Plate 18a, lower pore, edge of pore showing mycelial proliferation (arrowed). Bar = 10 µm.

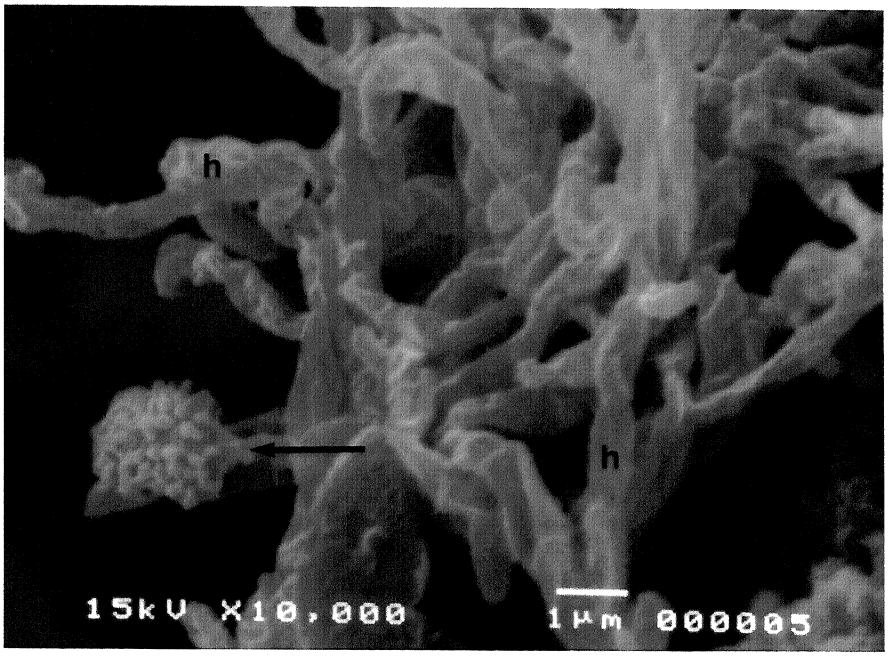

Plate 19b: Enlargement of Plate 19a, hyphae (h) and condidiophore (arrowed) with phialides, of similar size and shape to those of *Aspergillus sp.* Bar = 1 µm.

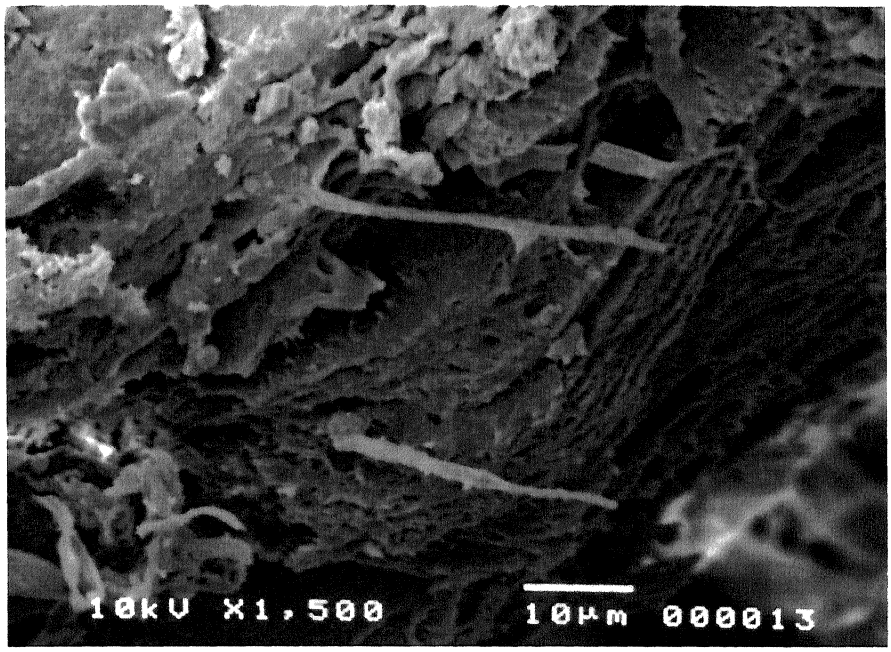

Plate 20a: Hypha extending from between calcite layers of shell.
Bar = 10 µm.

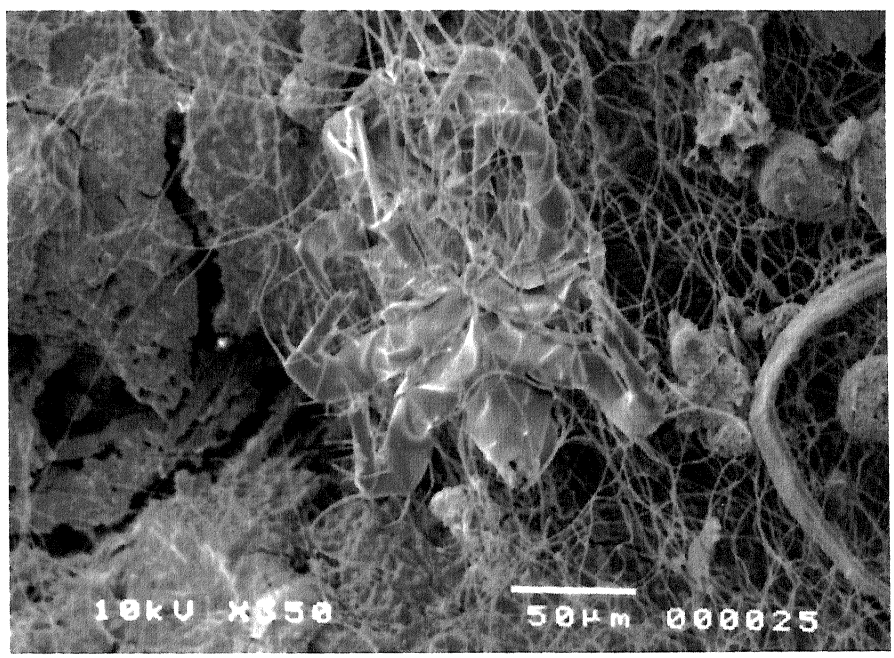

Plate 20b: Internal suface of shell showing mite colonised by hyphae.
Bar = 50 µm.

3.11 Trials

3.11.1 Nitrofurazone Treatment Trial

Results for the nitrofurazone treatment trial may be referred to in Table 19. These data have been presented in graphical form in Figure 2. Group A was treated with nitrofurazone. Group B had no treatment. Weights (in grams) have been calculated as the mean for each group based on a reducing number of survivors. Lengths have not been listed as the animals were only measured at the start of the trial and at four and eight week intervals.

Group	Duration weeks →	Start	1	2	3	4	5	6	7	8
A	Mean weight (g)	242	218	220	233	207	213	200	237	250
A	Std Dev	77	76	68	65	55	55	62	15	
A	N =	7	5	5	4	3	3	3	2	1
B	Mean weight (g)	296	305	307	297	253	247	259	320	325
B	Std Dev	92	90	65	58	45	56	67		
B	N =	7	7	6	6	3	3	2	1	1

Table 19: Results of nitrofurazone treatment trial (A = treatment, B = no treatment).

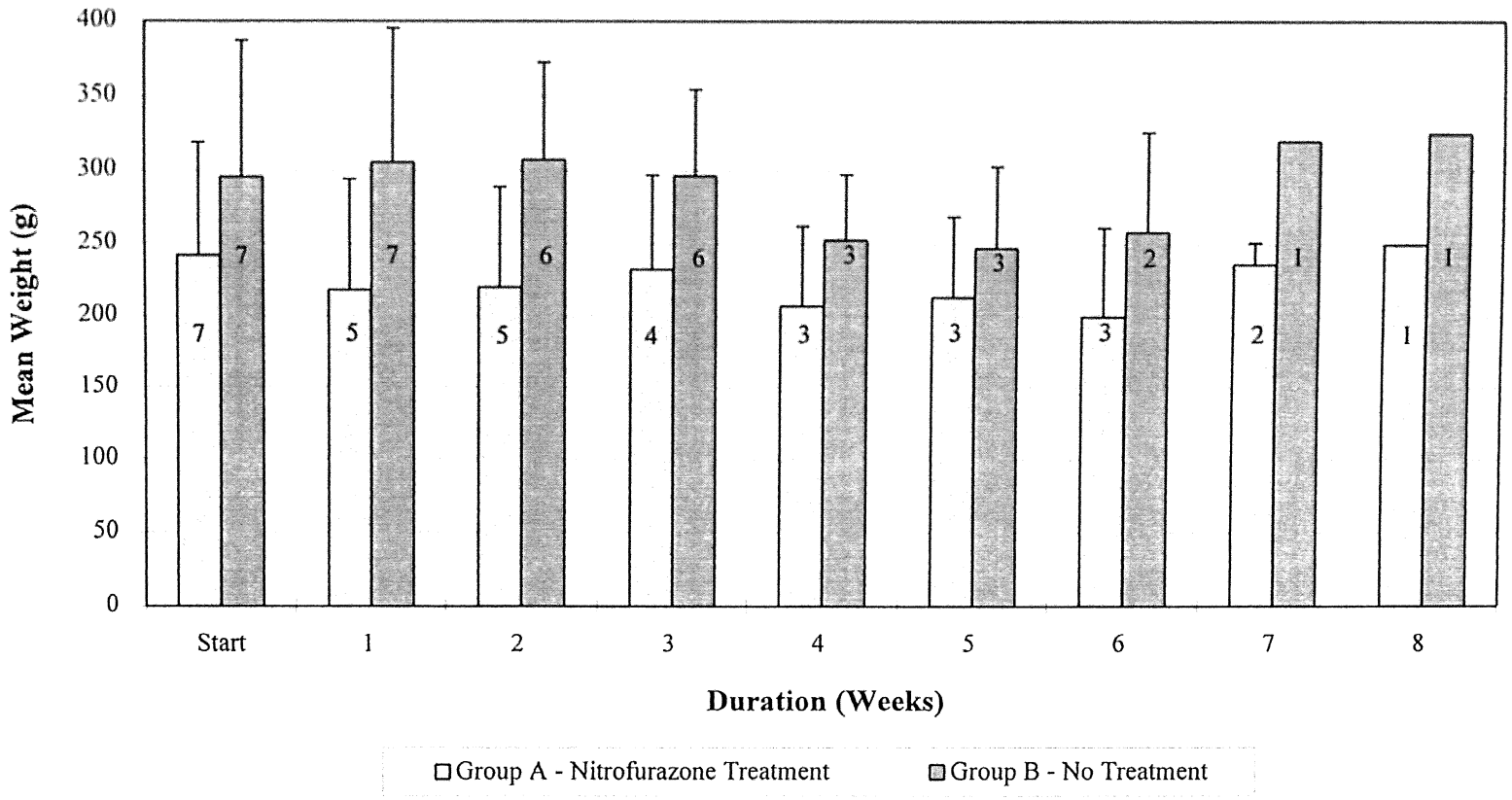

Figure 2: Results of nitrofurazone treatment trial by mean weight
(error bar = 1 std. dev., embedded numbers = survivors)

3.11.2 Thiabendazole Treatment Trial

Results for the thiabendazole treatment trial may be referred to in Table 20. The mean weights data have been presented in graphical form in Figure 3. Groups 6 and 7 were treated with thiabendazole. Group 3 had no treatment. Weights (in grams) and lengths (in mm) have been calculated as the mean for each group based on numbers of survivors.

3.11.3 Food Supplements Growth Trial

Results for the Food supplements trial may be referred to in Table 20. The mean weights data have been presented in graphical form in Figure 4. Group 1 received a basic diet of kangaroo meat with added calcium di-phosphate. Group 2 received kangaroo meat, calcium di-phosphate and Vita-Stress®. Group 3 received kangaroo meat, calcium di-phosphate, Vita-Stress® and Enzactiv®. Weights (in grams) and lengths (in mm) have been calculated as the mean for each group based on numbers of survivors.

3.11.4 Stocking Density Growth Trial

Results for the stocking density trial may be referred to in Table 20. The mean weights data have been presented in graphical form in Figure 5. Group 4 started with 10 animals in the pen. Group 5 started with 40 animals. Groups 6 and 7 both started with 20 animals in each pen. Weights (in grams) and lengths (in mm) have been calculated as the mean for each group based on numbers of survivors.

Combined results of mean weights for all seven groups in the thiabendazole treatment trial, food supplements trial, and stocking density trials are presented graphically in Figure 6.

		Weight (g)							Length (mm)						
	Group	Start	4 wks	8 wks	12 wks	16 wks	20 wks	24 wks	Start	4 wks	8 wks	12 wks	16 wks	20 wks	24 wks
Mean	1	54	72	104	144	192	228	249	293	314	344	381	410	426	445
Std Dev	1	13	23	45	57	68	87	104	22	26	39	46	46	51	58
N =	1	20	18	18	17	16	16	16	20	18	18	17	16	16	16
Mean	2	53	56	67	91	129	160	185	289	301	319	338	366	383	407
Std Dev	2	12	20	30	47	67	93	112	17	23	33	44	55	61	75
N =	2	20	20	18	18	16	16	16	20	20	18	18	16	16	16
Mean	3	56	53	54	69	91	115	133	292	296	308	321	337	351	369
Std Dev	3	13	19	21	37	59	84	106	22	25	30	38	51	62	73
N =	3	20	20	15	13	13	12	12	20	20	15	13	13	12	12
Mean	4	56	53	64	77	93	100	106	295	303	326	339	359	367	370
Std Dev	4	14	15	21	24	18	19	21	22	25	24	30	18	15	15
N =	4	10	10	7	6	5	5	5	10	10	7	6	5	5	5
Mean	5	59	58	62	78	105	132	153	300	311	327	339	360	377	395
Std Dev	5	12	18	24	30	41	58	76	19	23	26	32	36	46	55
N =	5	40	38	35	33	30	29	29	40	38	35	33	30	29	29
Mean	6	56	52	46	56	71	77	89	295	301	309	315	333	338	347
Std Dev	6	12	15	18	20	25	33	41	20	20	22	24	25	28	35
N =	6	20	19	17	14	11	11	10	20	19	17	14	11	11	10
Mean	7	53	48	41	50	63	69	76	290	292	294	307	318	325	329
Std Dev	7	11	10	9	9	8	13	18	23	24	23	17	11	13	15
N =	7	20	20	17	14	11	10	10	20	20	17	14	11	10	10

Table 20: Results of growth trials:- thiabendazole treatment, food supplements, stocking density.

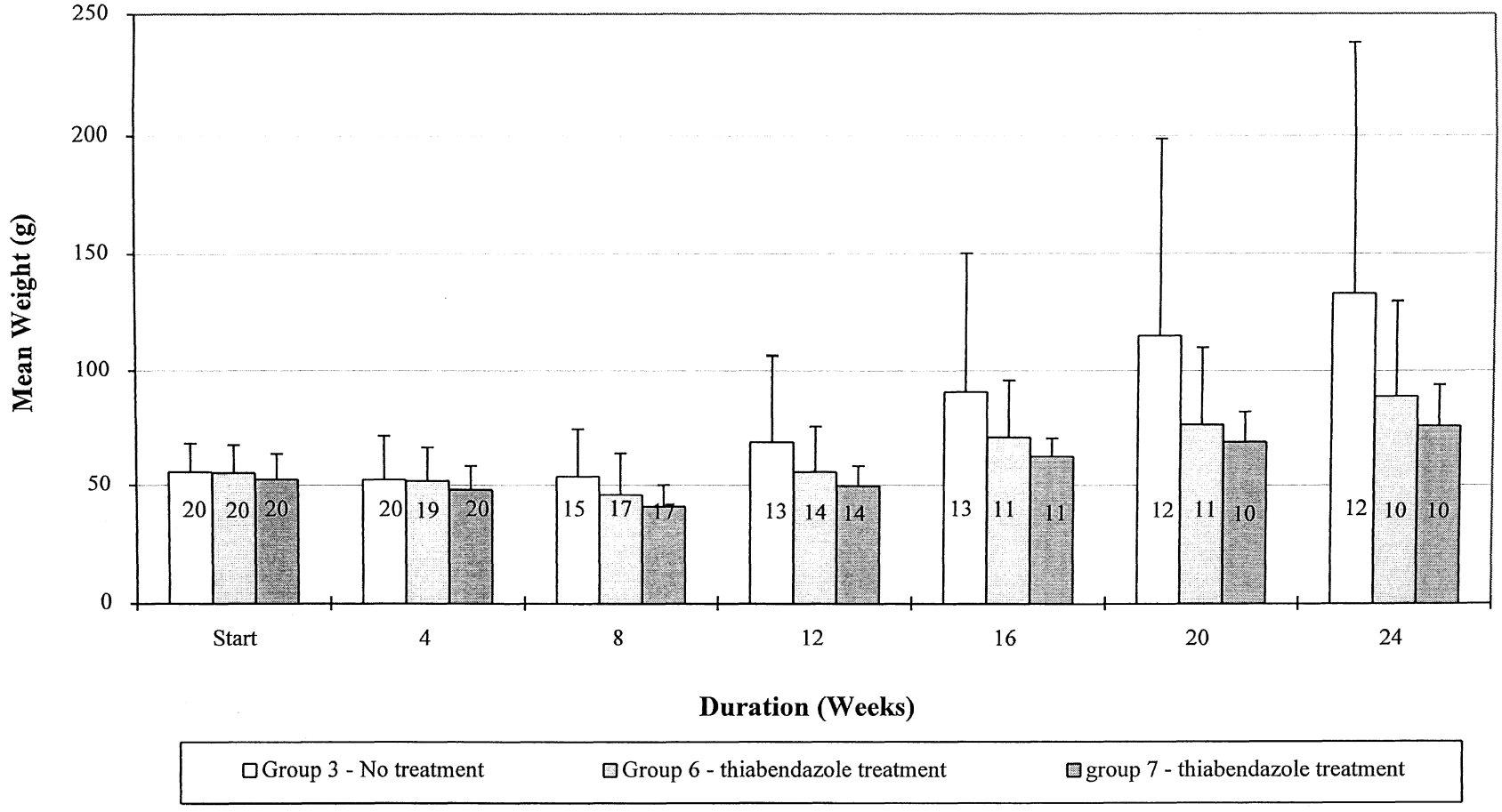

Figure 3: Results of thiabendazole treatment trial by mean weight
(error bar = 1 std. dev., embedded numbers = survivors)

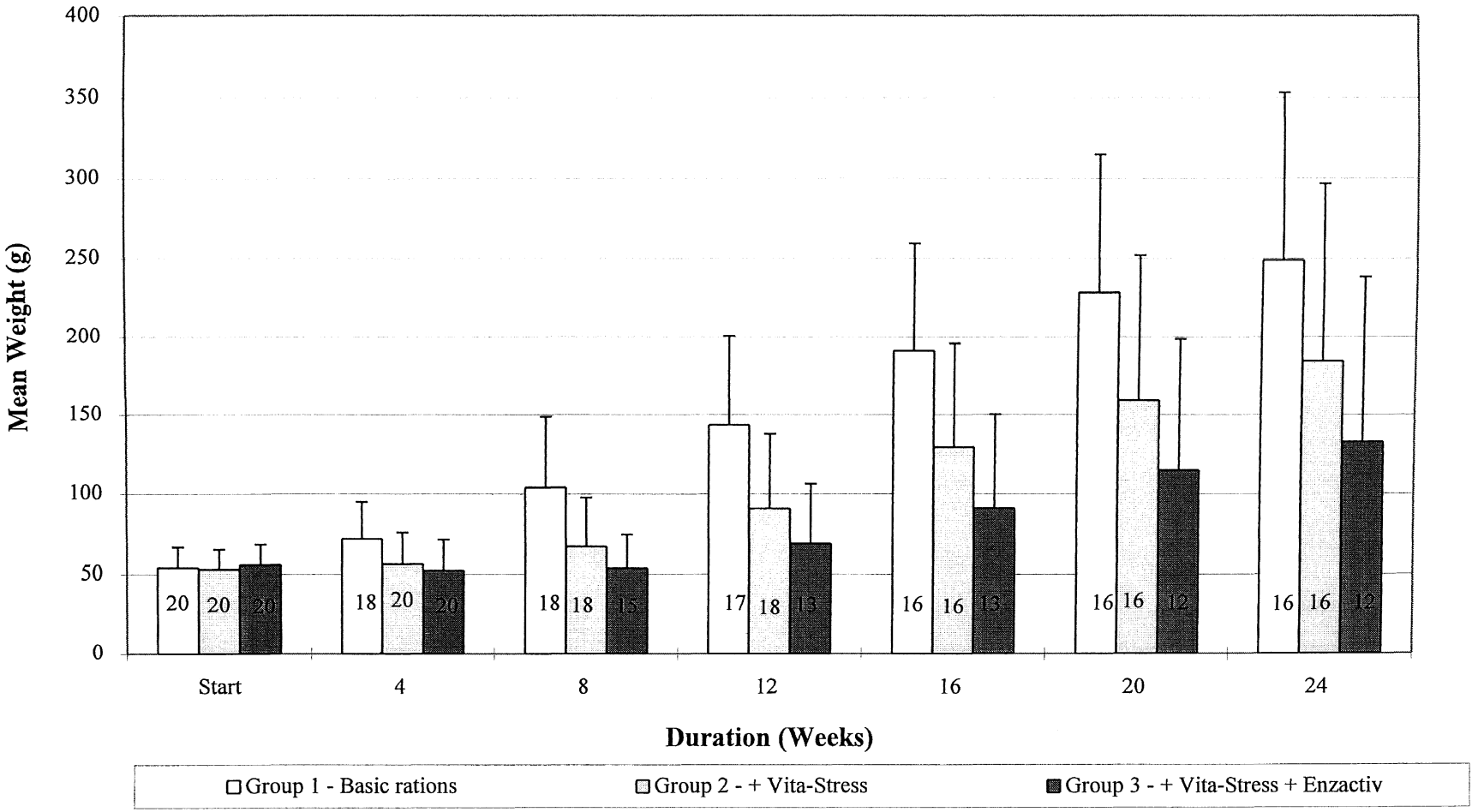

Figure 4: Results of food supplements trial by mean weight
error bar = 1 std. dev., embedded numbers = survivors)

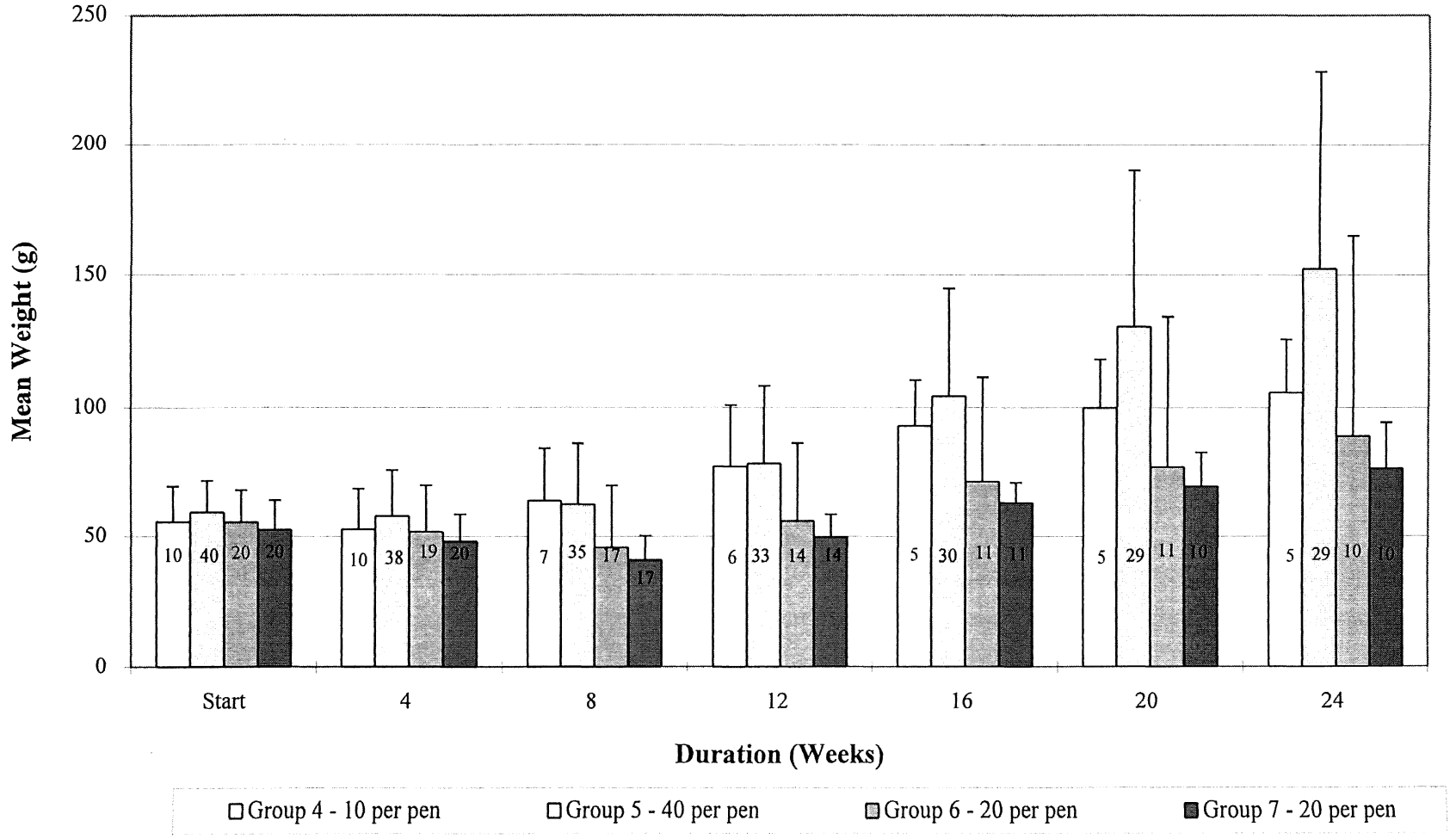

Figure 5: Results of stocking density trial by mean weight
(error bar = 1 std. dev., embedded numbers = survivors)

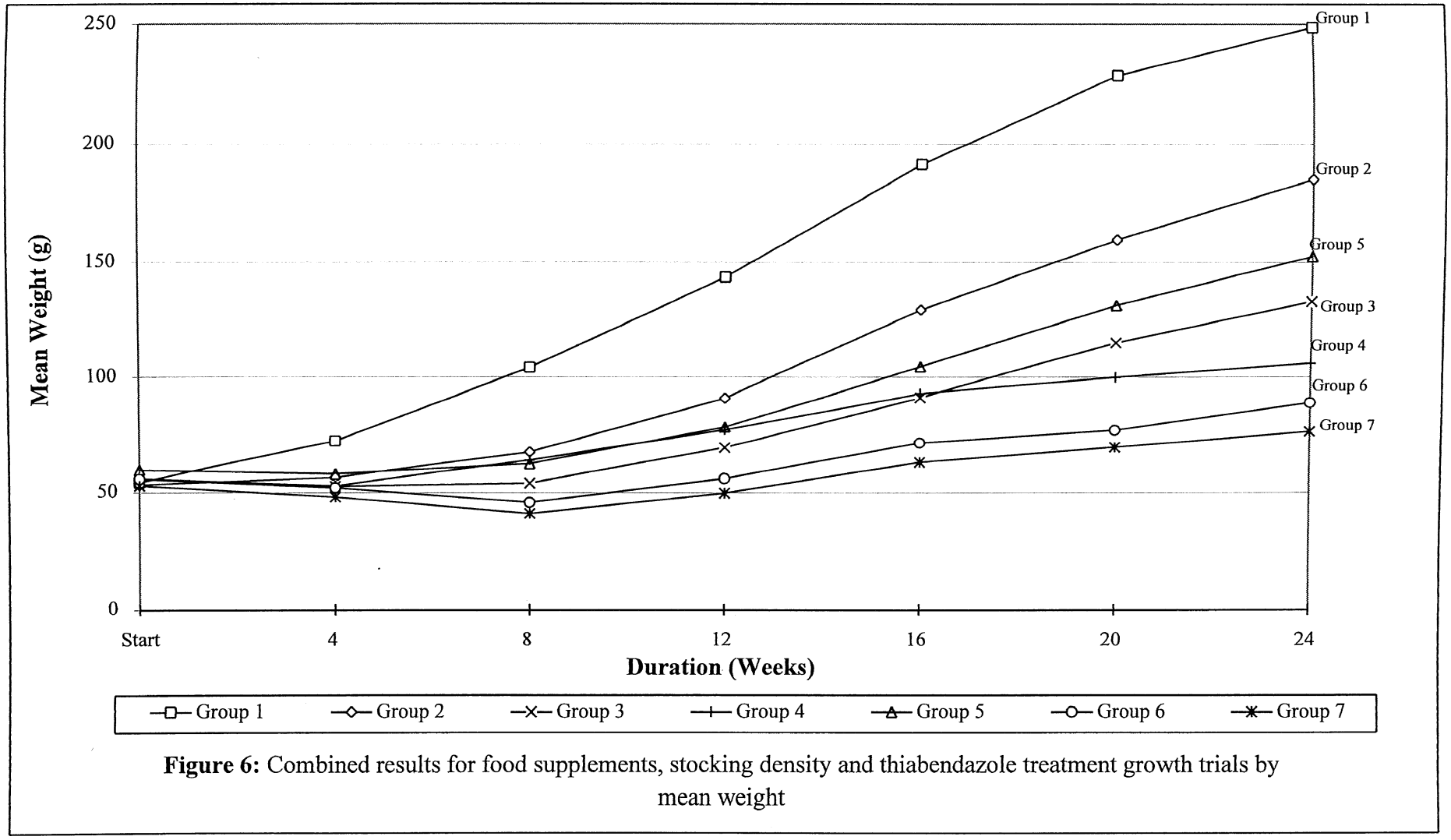

Figure 6: Combined results for food supplements, stocking density and thiabendazole treatment growth trials by mean weight

3.11.5 Light/Darkness Growth Trial

Results for the light/darkness trial may be referred to in Table 21. The mean weights data have been presented in graphical form in Figure 7 and mean lengths in Figure 8. Groups 1 and 3 were housed in darkness. Groups 2 and 4 were housed in diffuse sunlight. Weights (in grams) and lengths (in mm) have been calculated as the mean for each group based on numbers of survivors.

		Weight (g)				Length (mm)			
Grp		Start	1	2	3	Start	1	2	3
1-D	Mean	144	153	171	179	388	398	411	425
1-D	St Dev	29	30	43	55	26	26	33	41
1-D	N =	15	15	14	14	15	15	14	14
2-L	Mean	138	140	160	176	388	398	408	428
2-L	St Dev	31	33	46	60	29	31	36	43
2-L	N =	15	15	15	15	15	15	15	15
3-D	Mean	280	299	340	376	471	489	506	526
3-D	St Dev	78	83	104	132	39	43	47	54
3-D	N =	15	15	15	15	15	15	15	15
4-L	Mean	271	295	335	357	467	489	503	524
4-L	St Dev	84	81	98	117	38	39	43	46
4-L	N =	15	13	13	13	15	13	13	13

Table 21: Results of light/darkness growth trials (mean weight in grams, mean length in mm., D = darkness, L = diffuse sunlight).

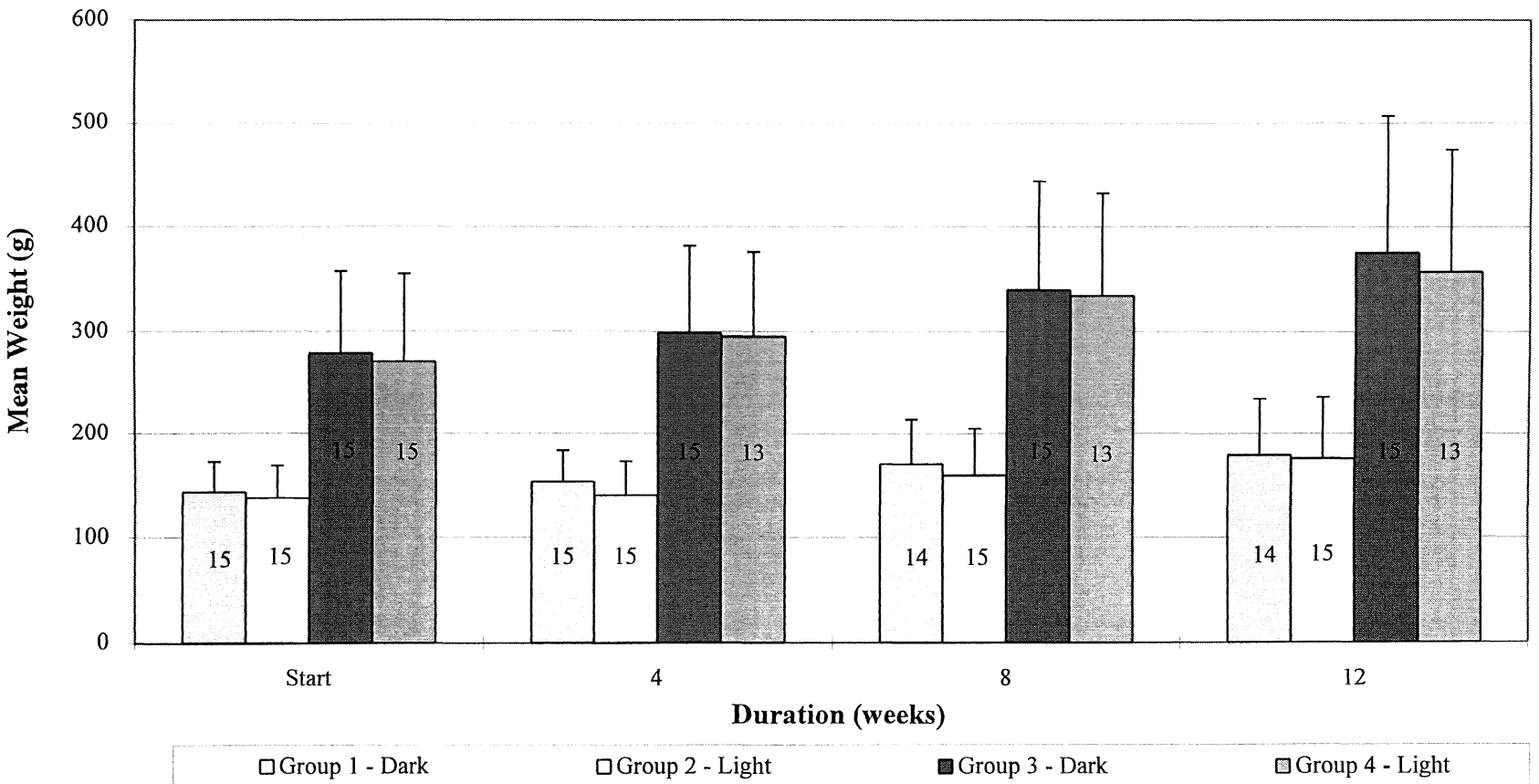

Figure 7: Results of light/darkness growth trial by mean weight
(error bar = 1 std. dev., embedded numbers = survivors)

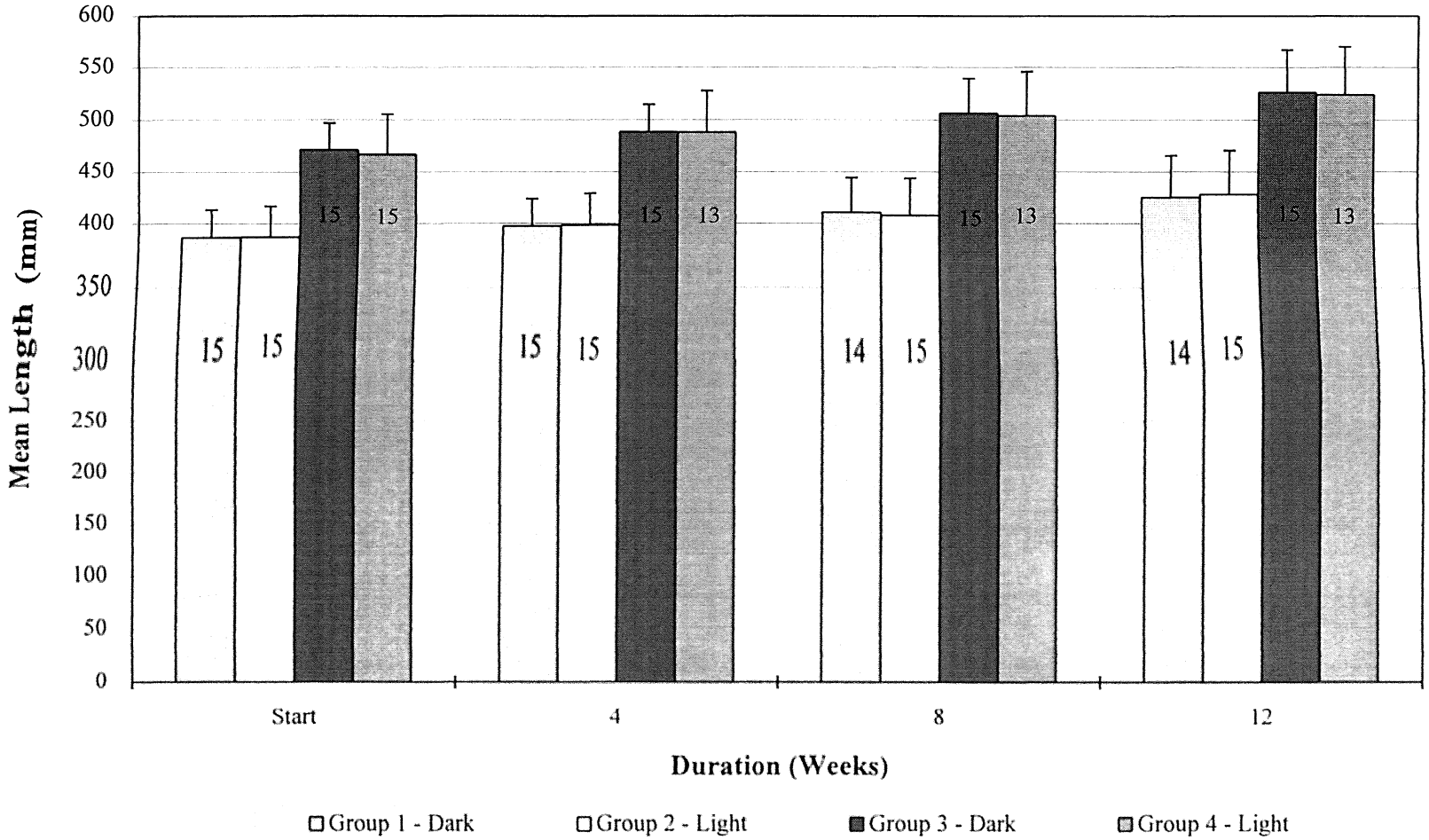

Figure 8: Results of light/darkness growth trial by mean length
(error bar = 1 std. dev., embedded numbers = survivors)

3.12 Egg and Hatchling Survival and Development

Egg and live hatchling production data are presented in Table 22. In Table 23 the data are expressed as percentages of the total eggs laid and also as percentages of fertile viable eggs. These data are represented in graphical form in Figure 9.

Hatchling survival data to the 31 December of each year from 1989 to 1994 inclusive are presented in Table 24.

Year	Total eggs laid	Damaged not set	Infertile eggs	Fertile eggs	Embryo deaths	Hatched
1984	40	**	**	**	**	13
1985	110	**	**	**	**	40
1986	600	**	**	**	**	110
1987	660	**	**	**	**	300
1988	1002	**	**	**	**	362
1989	1206	**	**	**	**	386
1990	1152	**	**	**	**	343
1991	1015	**	**	**	**	365
1992	1374	34	267	1073	702	371
1993	2015	68	407	1540	971	569
1994 to Nest 23	1098	34	141	923	259	664
1994 total, (32 nests)	1521	60	222	1239	476	763

Table 22: Egg and hatchling production chart. (** historical data not available)

	As % of total eggs laid					As % of Fertile
Year	**Damaged not set**	**Infertile eggs**	**Fertile eggs**	**Embryo deaths**	**Hatched**	**Hatched**
1984	**	**	**	**	32.5%	**
1985	**	**	**	**	36.4%	**
1986	**	**	**	**	18.3%	**
1987	**	**	**	**	45.4%	**
1988	**	**	**	**	36.1%	**
1989	**	**	**	**	32.0%	**
1990	**	**	**	**	29.8%	**
1991	**	**	**	**	36.0%	**
1992	2.5%	19.4%	78.1%	51.1%	27.0%	**34.6%**
1993	3.4%	20.2%	76.4%	48.2%	28.2%	**36.9%**
1994 to Nest 23	3.1%	12.8%	84.1%	23.6%	60.5%	**71.9%**
1994 total, (32 nests)	3.9%	14.6%	81.5%	31.3%	50.2%	**61.6%**

Table 23: Egg and hatchling production chart, as percentages (** historical data not available)

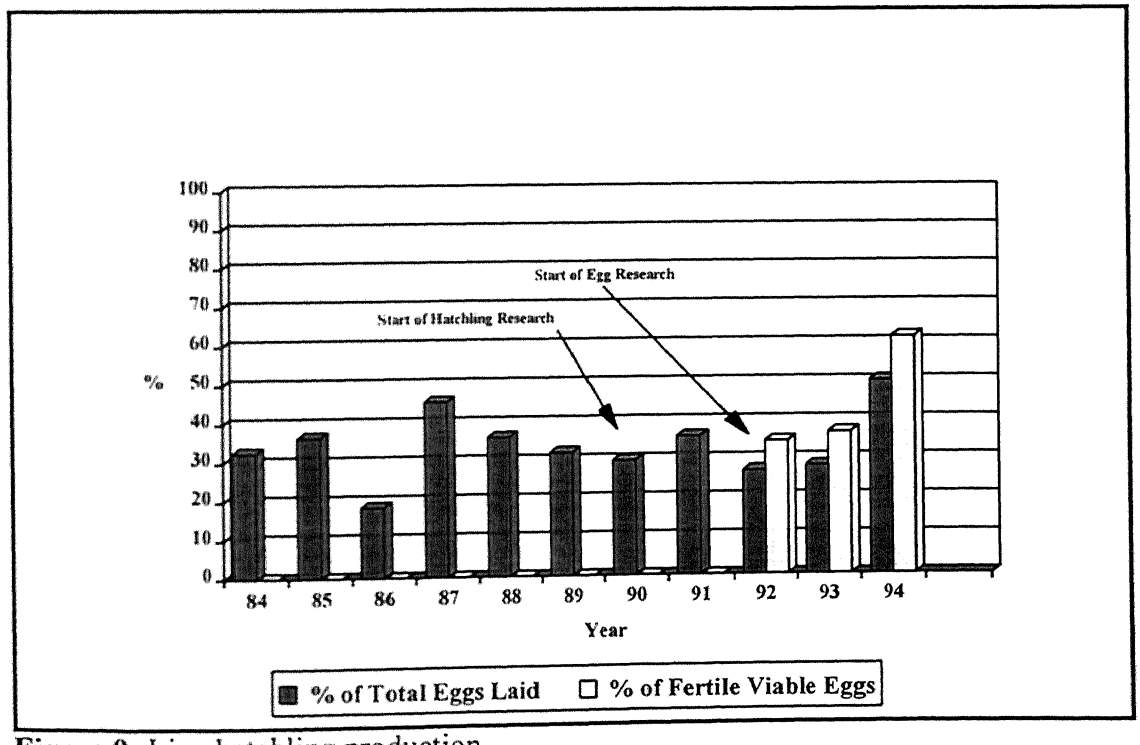

Figure 9: Live hatchling production

	30 June	31 Dec	31 Dec excluding unrelated deaths
1989	**	53.1%	**
1990	**	40.8%	**
1991	**	44.1%	**
1992	**	66.6%	77.4%
1993	88.8%	73.6%	78.9%
1994	**	82.9%	**

Table 24: Hatchling survival (** data not recorded)

3.13 Koch's Postulates

Eggs selected for validating Koch's Postulates hatched earlier than expected eleven days after experimental infection. None of the hatchlings showed any external lesions at hatching.

The twelve hatchlings used in the second trial and those hatched from experimentally infected eggs were housed similarly but in separate groups of three in Nally tubs. Escapes by some of the hatchlings from these tubs and subsequent intermingling of some groups and reduction of group numbers led to the scheduled time of euthanasia being cancelled. After observing for several months a selection of animals, which were thought to be still positively identified by group and treatment, were euthanased and post-mortems were carried out to investigate whether fungal infections were present. No external manifestations of fungal disease were apparent. Tissues sampled mycologically were heart, lung, liver, spleen, kidney. Results are shown in Table 25 and Table 26.

Initial treatment ↓ Inoculum→	Viable conidia	Non-viable conidia
Hatchlings, interperitoneal injection, 0.2 ml.	A + + - (17 wks)	B - - (15 wks)
Hatchlings, inhalation, 0.2 ml.	C	D + (17 wks)
Eggs, injection, 0.4 ml., hatched.	E + (19 wks)	F + (19 wks)
Eggs, dip 5 minutes, cool (~27°C), hatched.	G + (19 wks)	H - (19 wks)
Eggs, dip 5 minutes, warm (~32°C), hatched.	I + (19 wks)	J - (19 wks)

Table 25: Results of Experimental Infections to validate Koch's Postulates (Letters refer to groups' identification; + = fungal growth; - = no growth; each symbol indicates one animal; shaded block = no animal; bracketed figure is age of animals at euthanasia).

Hatchling identification	Tissue positive to fungal growth.
Group A, animal 1	lung, liver
Group A, animal 2	lung
Group A, animal 3	none
Group B, animal 1	none`
Group B, animal 2	none
Group D, animal 1	kidney
Group E, animal 1	lung
Group F, animal 1	lung, liver
Group G, animal 1	liver, kidney
Group H, animal 1	none
Group I, animal 1	lung
Group J, animal 1	none

Table 26: Results of mycological sampling in validation of Koch's Postulates.

4. DISCUSSION

Mycoses in farmed crocodiles have become more commonly reported (refer section 1.4.1). Establishing cures for such diseases is difficult due to the resistance of the pathogens and the highly stress-sensitive nature of the hosts. There is no reason to believe that eggs and hatchlings produced in the wild do not succumb to fungal disease as do farm reared stock given that the natural habitat of the crocodile host and the fungal pathogen are so similar. Both crocodiles and fungi thrive in warm humid conditions and the nesting materials used by both farmed and wild crocodiles (*ie.* soil, sand, leaf litter, decomposing plant material, and other organic substrates) are the normal environment in which to find fungi. Pooley and Ross (1989) reported that not only does predation, desiccation, drowning due to flooding of the nest, damage by other laying crocodiles, high or low temperatures and noxious gases in the nest contribute to egg mortality in the wild, but also that "excessive dampness in the nest can encourage fatal fungal growths on the eggs". No further details were given regarding fungal species involved. Magnusson (1982), in an investigation of egg mortality of *C. porosus* in northern Australia, stated that "other causes of mortality of eggs, such as fungus infections or inappropriate temperature regimes due to poor nest construction, undoubtedly occur", However, other factors in that study prevented further investigation.

Although wild crocodiles frequent the waters of the Fitzroy River and were known to have nested prior to the commencement of this study, no nests were located in regular surveys by Koorana Crocodile Farm during the period of this research (Lever, pers. comm.). Hence, the opportunity for sampling nesting material and eggs in the wild did not arise. Crocodile farmers in the Northern Territory are given permits to collect limited numbers of *C. porosus* hatchlings and eggs from the wild (Webb, Whitehead and Manolis, 1987). It is understandable, however, that only viable undamaged eggs and healthy hatchlings are collected for raising in captivity (O'Brien, pers. comm.). Unexplained developmental failures were one of several reasons for *C. porosus* egg mortality in the wild (Webb, Whitehead and Manolis, 1987) with mean egg survivorship estimated at about 25% of eggs laid (Webb, Whitehead and Manolis, 1987, Webb and Manolis, 1989). This high egg mortality will remain unexplained until more research is carried out to determine whether fungi are causal agents of or

are contributing factors to such failures. As mentioned previously Pooley and Ross (1989) and Magnusson (1982) have intimated that this is the case.

Predators and scavengers quickly remove sick and dead wild animals (Wobeser, 1994). Therefore, any weak diseased crocodile hatchlings in the wild would quickly fall prey to their natural predators. The hatchlings' small size would also make their discovery and collection difficult and, therefore, fresh diseased-affected carcasses suitable for post-mortem would be a rarity. Annual survivorship of hatchling *C. porosus* to one year of age has been estimated at 54% (Webb, Whitehead and Manolis, 1987, Webb and Manolis, 1989).

Hatai and Egusa (1978) and Hatai *et al* (1978) in their studies of *F. solani* causing black gill disease in the cultured Kuruma Prawn, *P. japonicus*, suggested that the source of the pathogen was the sand in the culturing ponds. Fungal elements (conidia, chlamydospores and mycelia) of *F. solani* had been isolated from the ponds holding diseased prawns but not from healthy prawn ponds. *F. solani* was also isolated from wet sand in ponds which had been empty for two months.

Similarly in the situation reported here *F. solani* and other fungi were cultured from the soil, natural nesting material, and from the air at the crocodile farm, and consequently from walkways, pens and work areas after being moved by natural air currents or by human or animal movement. Since the conidia were ubiquitous in the environment, total elimination of them would be impossible. A reduction in conidial frequency in the animal housing areas was enabled by disinfection of the appropriate surfaces, use of footbaths, and change in water supply from well-water to reticulated town supply.

Many of the reported fungal infection in reptilians are caused by opportunistic fungi. A fungal organism may seize the opportunity to invade tissue that has already sustained some physical or chemical trauma (Frye, 1991). This may be true in some of the crocodiles in this study with the possible route of entry through wounds caused by other crocodiles during the scramble at feeding time, dominance fights between large and smaller animals, or skin abrasions from the concrete in the pens.

Muhvich *et al* (1989), reporting *F. solani* mycoses in captive newborn Bonnethead sharks, also suggested that skin trauma was a possible site of infection, and that

abrasion of the nares or skin against the cement surface of their holding tanks was the probable cause of the trauma in the sharks.

A reduction in wounds in the juvenile crocodiles may have been achieved by the addition of extra feeding sites, provision of extra 'hides' as shelter for less aggressive animals, and the more frequent grading of animals according to size. Concrete pens constructed after the completion of experimental work for this study were modified in order to lower the incidence of skin abrasions.

Although the possibility exists that infection of the hatchlings was by the respiratory route, this is not considered likely since aerosols rarely occurred in the hatchling pens or buildings. A change of water supply, and additional cleaning and disinfection regimes reduced the incidence of inoculum in the water. There was no manual aeration of the water other than that caused during occasional scuffles between hatchlings, therefore little or no aerosolisation of any pathogen from the water would have occurred. Hatchlings and yearling were housed permanently indoors so windborne conidia projected into the moving air mass, perhaps after an initial 'rain splash' event, were also considered as a possible but not probable source of respiratory infection. Attempts at airborne infection of terrapins, using samples of *P. lilacinus* and *M. anisopliae* isolated from crocodiles, were not successful (Austwick and Keymer, 1981). This possibly suggests, therefore, that this was not the usual natural method of infection in that instance.

One of the factors which influences the success of any disease management program is the nature of the causative agent (Wobeser, 1994). There are two groups of agents, endogenous and exogenous. Endogenous agents may be ubiquitous healthy inhabitants in the external environment and are often present in the body without causing obvious disease. Endogenous agents normally only induce disease under particular circumstances, *eg.* when the host is stressed. These types of agent are commonly termed opportunistic or facultative pathogens and include the fungus *A. fumigatus* (Wobeser, 1994). *F. solani* is also classified as an opportunistic pathogen as is *P. lilacinus* (Rippon, 1988). Exogenous agents are not present in the bodies of healthy animals but are acquired from external sources *eg.* rabies virus (Wobeser, 1994). Exogenous agents do not normally form persistent chronic infection in the host, but produce a well-defined disease within a predictable time after introduction,

and mostly do not survive in the external environment (Wobeser, 1994). The disease processes reported from this study are of the endogenous type, *ie.* opportunistic infections caused by pathogens ubiquitous in the environment which have become infective due to stresses on the embryo or hatchling crocodile.

Chronic stress is by definition stress that persists for an extended period of time (Lance, 1994). Where chronic stress in reptiles has been identified the results are loss of body mass, reproductive failure and increased susceptibility to disease (Lance, 1992 cited in Lance, 1994). Classic examples of causes of chronic stress in reptilian species include overcrowding, exposure to inappropriate temperature and light levels (Regal, 1980 in Lance, 1994), noise, inappropriate humidity, an inappropriate social situation, and inappropriate nutrition (Lance, 1994). Acute stress has an immediate phase, the 'fight or flight' response to a sudden threat or loud noise, and a second slower phase in which physiological and hormonal changes occur over several hours (Lance, 1994). The chronic stress of prolonged exposure of farmed *C. niloticus* to low temperatures has been shown to cause suppression of the immune system and increased susceptibility to disease (Foggin, 1987). In investigations by Penrith *et al* (1991) it was suggested that pox virus infection had been present in a latent but sub-clinical form in quarantined juvenile caimans (*C. crocodilus fuscus*) and that the disease had undergone clinical manifestation and/or recrudescence due to the stress of a changed environment (*ie.* international travel). Lack of sunlight as well as disturbances due to staff activities were also considered as being complicating stress factors (Penrith *et al,* 1991). In the study of systemic fungal infection in *C. niloticus* Huchzermeyer (1991) reported that few of the crocodiles developed terminal lesions in the lungs and liver, but the incidence of mild infections was thought to be high with the animals' immune systems affected by the chronic stress of prolonged cold.

Similarly, in the study reported here, the crocodile hatchling mycoses may be long term latent infections initially received while in the egg. The mildly affected animals show few or no visible symptoms and may continue to grow until internal organ damage results in terminal disease. External symptoms are not manifested until precipitated by either a series of acute stresses (short term) or by one or more chronic stresses (long term) - or a combination of both.

Haematological and biochemical test had been carried out to determine if there were any physiological changes in the crocodiles which were attributable to stress.

Canfield (1985) in a characterisation study of crocodilian blood cells presented haematological values for four *C. porosus* and four *C. johnstoni.* Comparable parameters from that study are summarised in Table 27.

Age and sex	2-4 yrs, not sexed							
Species	*C. porosus*, animals 1 - 4				*C. johnstoni*, animals 5 - 8			
Hct (%)	22	21	20	20	18	20	21	20
RBC ($x10^{12}$/L)	0.93	0.86	0.92	0.98	0.71	0.89	0.93	0.82
Hgb (g/L)	67	63	62	77	57	70	75	68
WBC (x 10^9/L)	39.6	44.2	41.2	41.8	39.2	26.4	34.6	48.8

Table 27: Summary of haematological findings of Canfield (1985) for *C. porosus* and *C. johnstoni.*

Attempts to quantify total erythrocyte and leucocyte levels on campus were not successful. This was most likely because the blood sample was outside its useful storage life. According to Diagnostica MERCK (1984), EDTA-blood may be stored for up to seven days at room temperature if it is to be used for haemoglobin determinations, but only for up to twenty four hours for erythrocyte and leucocyte counts. Thrombocytes (or platelets) are only stable for up to two hours at 4°C. Blood samples which are to be used for smears and subsequent differential blood cell counts should be used within two to four hours and stored at room temperature. The blood samples as used were approximately twenty four hours old when returned from the pathology laboratory and had been stored at 4°C in the interim. Cell damage may have already occurred by this stage.

Electronic recording instruments may give false high readings for low thrombocyte counts (Diagnostica MERCK, 1984). As all crocodilian erythrocytes, leucocytes and thrombocytes are nucleated, unusually high readings will also result, therefore an automatic blood counter should not be used. Erythrocyte numbers can, however, be approximated by this method (Frye, 1986). As the blood samples processed by the Pathology Laboratory (A) were processed in an automatic Coulter SPlus4 some of the

results listed in Table 15 (refer section 3.4) may be spurious. This machine was calibrated for human blood cell quantification which means some readings (*eg.* MCH and RDW) were outside the normal human range and could not be determined.

Referring to Table 15 in section 3.4, haematocrit values for animals 1 (23.4%) and 3 (23.2%) in this study are similar to those of Canfield (1985). However, animal 2 had a higher reading (30.9%), which may possibly indicate dehydration. The animal did not however appear to be dehydrated, as it's soft belly skin was neither wrinkled nor flaking. All 6 animals had been housed in humid indoor pens with water freely available. Their period of restraint away from that environment was less than two hours. Animals 5 and 6 by comparison had low haematocrit values (11.7% and 17.7%) which may indicate vitamin deficiencies, anaemia or liver degeneration. As lower haematocrit values often occur in juveniles, these readings may in fact be in the normal range for hatchling crocodiles. Haematocrit values for *C. niloticus* (Foggin, 1987) were normal mean 22% (range 13-27%) and runt mean 13%. Morpurgo *et al* (1992) reported a range of 19.5-21.5% for twelve to eighteen month old *C. niloticus*. Haematocrit values for resting captured wild *C. porosus* (Seymour *et al*, 1985) were mean 22.2% and range 19.8-26.3%. Grigg and Cairncross (1980) reported a haematocrit mean of 24.8%, range 19.1-31.3% for 96 wild *C. porosus* (age not specified), similar to the results in this study for animals 1 and 3, with the animal 2 result at the upper limit of their reported range. Haematocrit for two to four year old *Caiman latirostris* (Tourn *et al*, 1994) was reported as 20.5% for females, 17.19% for males and 19.52% overall, similar to values for *Crocodylus rhombifer* (Carmena-Suero *et al*, 1979 *in* Tourn *et al*, 1994). Wallach (1969) combining data from several authors reported a haematocrit range for all crocodilians of 16-29%.

RBC values for animal 1 (0.95 x 10^{12}/L) and 3 (0.94 x 10^{12}/L) are similar to those of Canfield (1985) for *C. porosus* as listed in Table 27, with animal 2 (1.24 x 10^{12}/L) having a higher value possibly due to dehydration. The low values for the two sick animals 5 (0.53 x 10^{12}/L) and 6 (0.78 x 10^{12}/L) may be age related or may indicate anaemia. Wallach (1969) combining data from several authors reported a lower RBC range for all crocodilians of from 0.331 x 10^{12}/L to 0.676 x 10^{12}/L. Wintrobe (1933) cited in Reichenbach-Klinke and Elkan (1965b) reported the RBC value for *A.*

mississippiensis of 0.67 x 10^{12}/L. Jacobson (1988a) published a RBC range for *A. mississippiensis* of 0.618 x 10^{12}/L to 1.48 x 10^{12}/L.

The investigations reported here show higher haemoglobin levels (104-140 g/L) for the three healthy animals than those reported for *C. porosus* by Canfield (1985) (refer Table 27), with the results for two of the sick animals (51 and 91 g/L) more closely matched to the results in that report. High Hgb is normally caused by dehydration and shock. However, Frye (1986) reported a haemoglobin range of 59-120 g/L as representative for the alligator. Seymour *et al* (1985) reported resting *C. porosus* haemoglobin levels of mean 70.9 g/L and range 67.5-76.6 g/L. Jacobson (1988a) published haemoglobin levels of 71 g/L for *A. mississippiensis*, 86 g/L for *Caiman* sp., and 90 g/L for *C. acutus*. Haemoglobin values for *C. niloticus* (Foggin, 1987) were normal mean 74 g/L (range 64-87 g/L) and runt mean 39 g/L. Wallach (1969) combining data from several authors reported a haemoglobin range for all crocodilians of 50-92 g/L.

Total leucocytes could not be measured as the count was outside the machine's determinable range.

Jacobson (1988a) reported that both haematological and serum biochemical values in reptiles vary tremendously with age, reproductive state, season, body temperature, nutritional state, and disease state. Jacobson (1988a) also reported that although information is available on these values, few of the studies have been conducted under standardised conditions. The data, therefore, become difficult to interpret and compare, particularly when different methods of reporting results are used. In relation to the six animals available for blood sampling at the time of this study, age (three years old *cf* twelve months old), body temperature (affected by differing body size and being restrained), nutritional state (healthy appetite *cf* anorexic), and disease state (healthy *cf* infected by fungus) would all, therefore, have had an effect on the results presented.

Biochemical tests on blood serum were performed initially at a pathology laboratory using instruments calibrated for the determination of human blood biochemistry. Therefore, where crocodilian levels were outside the normal human range for a particular parameter, the result was recorded as, for example, >0.71 mmol/L for uric acid, and >3.9 mmol/L for phosphate. The results recorded by a veterinary pathology

laboratory (blood received and tested after six days) varied from those recorded at the other laboratory (processed on the day of sampling). This could be attributed to the age and deterioration of the blood samples. Haemolysis of the blood samples will also affect many biochemical tests. Interpretation of biochemical results was made with reference to Mitruka and Rawnsley (1977) and Kee (1995).

The reading for albumin from pathology laboratory A, allegedly outside the determinable range (<1.0 g/L) may be an error in recording as both laboratories reported albumin as g/L, and both sets of results for total serum protein (also in g/L) were very similar. Albumin normally makes up more than half the total protein and is synthesised by the liver. Albumin values for *C. niloticus* (Foggin, 1987) were normal mean 19 g/L (range 15-23 g/L) and runt mean 15 g/L. The A/G ratio is a calculation of the distribution of the two major protein fractions, albumin and globulin with low A/G ratios occurring in liver and renal disease. Low albumin levels occur in cases of severe malnutrition and malabsorption, consistent with animals 4, 5 and 6, which were anorexic. Albumin levels would normally be higher in juveniles. Decreased total protein levels occur in cases of prolonged malnutrition, starvation, malabsorption syndrome, severe liver disease, and chronic renal failure, all of which may have been a factor in the health status of animal 5. Total plasma protein levels in the crocodiles used in the study by Canfield (1985) were 48-70 g/L for the four *C. porosus*, and 33-69 g/L for the four *C. johnstoni*. Jacobson (1988a) published total plasma protein levels of 51 g/L for *A. mississippiensis*, 59 g/L for *Caiman* sp., and 65 g/L for *C. niloticus*. Total plasma protein values for *C. niloticus* (Foggin, 1987) were normal mean 53 g/L (range 46-85 g/L) and runt mean 42 g/L. Although this study used blood serum to obtain total protein levels, these results are comparable with total plasma protein levels and frequently the terms plasma and serum are used interchangeably in blood biochemistry. Except for animal 5, the results reported here are within the range reported by others.

The very low globulin level in animal 5 (19 g/L) may suggest severe liver disease (*eg.* mycotic infection, as was present at post-mortem in animals from the same group) or haemolytic anaemia, which correlates with low erythrocyte count for this animal. Gamma globulins are the body's antibodies which contribute to immunity, so low globulin levels support the theory that the sick animals were immuno-compromised.

Globulin values for *C. niloticus* (Foggin, 1987) were normal mean 31 g/L (range 22-39 g/L) and runt mean 30 g/L.

The differing values in total bilirubin between the healthy and sick animals may be in fact due to the age difference. Newborn levels are normally very high, with juvenile values dropping to levels lower than the normal range for adults. Decreased total bilirubin is normally the result of iron deficient anaemia (possible in the sick crocodiles) or from the effect of certain drugs (not possible in these animals). Total bilirubin and creatinine levels are decreased by exposure to light (both sunlight and artificial), which could explain the differences in results between laboratories A and B. A high fat food intake within twenty four hours prior to blood sampling may raise bilirubin levels. Excess fat was normally trimmed from any meat fed to juvenile crocodiles, therefore, although higher bilirubin levels were determined in animals 1, 2 and 3 which were eating normally, those levels should not have been affected by fat content in their diet. Animals 4, 5 and 6 were anorexic. Serum creatinine levels may be elevated due to a diet rich in creatinine, for example beef which was feed daily to the crocodiles and readily consumed by the healthy animals. Creatinine values also increase with age and or muscle mass as would be the case with the large healthy well muscled three year old crocodiles.

Urea levels in the three sick twelve month old animals were much lower than those of the two healthy three year old females. The male urea level was also lower than the female but not as low as the younger animals. Urea is converted from ammonia, a by-product of protein metabolism in the liver. High ammonia levels or low urea levels are an indication of severe liver disease. In humans blood ammonia is highest in the newborn, with lower levels in juveniles and still lower levels in adults. The urea levels found in this study may lie within the normal ranges for the relevant *C. porosus* juvenile age groups. Jacobson (1988a) published an urea level of 0 mg% for *A. mississippiensis* and Frye (1991), from an amalgamation of multiple cited sources, similarly quoted 0 mg/dL for the same species but neither author gave any indication of the age class of the alligators used in those studies.

CPK is an enzyme found in high concentration in the heart and skeletal muscles and low concentrations in the brain. Levels vary between male (higher) and female, and between newborn (highest), juveniles (lowest) and adult. Elevated levels may be

caused by skeletal muscle disease, vigorous exercise and trauma. The high levels for animals 1 and 6 may be associated with stress and may have been induced by the method of blood sampling and the period of restraint before blood letting. The very high level for animal 6 may indicate muscle damage which was consistent with it's recent history of wasting. The CPK levels found in this study may, however, lie within the normal ranges for the relevant *C. porosus* juvenile age groups.

The enzyme GGT is primarily found in the liver and kidney with smaller amounts in the spleen and heart. GGT levels vary between male (higher) and female, and between newborn (high) and adult. Juvenile levels are similar to adult levels. The high level reported for animal 6 from veterinary pathology laboratory B may be an error in recording as this test was not performed by this laboratory for the other 5 animals. Otherwise the readings from pathology laboratory A do not show much variation. Elevated levels may indicate liver or kidney disease.

AST is an enzyme found mainly in the heart muscle and liver, and high levels of serum AST occur following heart and liver damage. Juvenile and adult levels are similar and exercise tends to increase values. The values recorded may be within the normal range for *C. porosus*. This enzyme is normally compared with CPK and LDH. AST values (presented as SGOT, Serum glutamic oxaloacetic transaminase) values for *C. niloticus* (Foggin, 1987) are normal mean 16.6 IU (range 6.7-22.7 IU) and runt mean 42.7 IU.

High GLDH levels indicate liver cell lysis with most animal species having an acceptable range of 0-20 IU/L. The levels recorded may be normal for *C. porosus*.

Alkaline phosphatase is an enzyme produced mainly in the liver and bone with lower levels in adults. Decreased levels indicate malnutrition, consistent with animal 5 (anorexic and wasting). High levels are normally found in growing juveniles because of bone growth.

Amylase is an enzyme derived from the pancreas, salivary gland and the liver. Decreased levels may indicate necrosis of the liver, possible in animal 4 and 5.

ALT is an enzyme found primarily in the liver and elevated levels indicate liver disease. Juvenile levels are normally similar to those of adults. Decreased levels may be induced by exercise. This may be the reason for the very low ALT reading for animal 3 (<5 IU/L) due to its struggling against its physical restraints. All six

crocodiles had been tied up for at least an hour by the time of blood sampling, with the sick and weak animals lying quietly, but the healthy more active animals periodically attempting to break free. There was insufficient blood sample from animal 6 for ALT and amylase tests. ALT values (presented as SGPT, Serum glutamic pyruvic transaminase for *C. niloticus* (Foggin, 1987) are normal mean 13.1 (range 9.0-20.4) and runt mean 34.8 IU.

LDH for all animals was not able to be recorded due to insufficient blood samples. Elevated levels may be caused by shock, skeletal muscle disease and heat stroke. Levels recorded for the two female three year olds may be normal for *C. porosus*.

Uric Acid is a by-product of purine metabolism with meat and poultry (fed daily to the crocodiles) having high levels of purine. Excess quantities of uric acid are normally excreted in urine. However, continually elevated levels lead to urate deposits in tissues and synovial fluid of joints. Jacobson (1988a) published a reported uric acid level in *A. mississippiensis* of 3 mg% (1.98 mmol/L), and Marcus (1981) reported the normal range of uric acid levels in *A. mississippiensis* to be 1.0-4.1 mg/dL (0.66 to 2.7 mmol/L) in blood plasma, much higher than the animals tested here. Uric acid values for *C. niloticus* (Foggin, 1987) were mean 4.1 mg%, range 1.4-7.5 mg%, (mean 2.7 mmol/L, range 0.92-4.9 mmol/L). There was insufficient blood sample from animal 6 for uric acid testing. Prior to the start of this research, gout had been suspected in some animals which had reduced usage of the fore-limbs (Lever, pers. comm.). However, histological analysis found no indication of this (Hunt *et al*, 1988). Gout has been reported in *C. niloticus* (Foggin, 1987), *A. mississippiensis* (McNease and Joanen, 1981 *in* Foggin, 1987), *C. johnstoni* (Buenviaje *et al*, 1994), *Crocodylus* sp. (Youngprapakorn *et al*, 1994) and *C. novaeguineae* (Ladds *et al*, 1995).

Serum CO_2 acts as a bicarbonate determinate. When serum CO_2 is low, bicarbonate is lost and metabolic acidosis may result. A decreased serum CO_2 may indicate dehydration, shock or over-exercise. The low levels found in the six animals in this study (7-15 mmol/L), particularly in two of the older healthier crocodiles may be related to the stress of being restrained and struggling against physical restraints. Seymour *et al* (1985) reported that bicarbonate levels in juvenile *C. porosus* decreased within 10 to 20 minutes when exercised to the point of exhaustion. They reported resting bicarbonate values for juvenile *C. porosus* in the range 20.0 to 26.2 mmol/L.

Grigg and Cairncross (1980) reported plasma bicarbonate levels of 16 mmol/L in two captive adult *C. porosus* and 17 mmol/L in 99 wild-caught *C. porosus*. Juveniles and adults normally have similar ranges of serum CO_2.

Glucose is formed from dietary carbohydrates and is stored as glycogen in the liver and muscles. Insulin and glucagon, two pancreatic hormones, affect the blood glucose level. Elevated levels may be caused by stress, exercise and hypothermia, all possibly affecting the six animals sampled. Low levels may result from malnutrition as in animals 4 and 5 which were anorexic, or from hypoglycaemic reaction due to an excess of insulin. Hypoglycaemia is discussed later in this chapter. Jacobson (1988a) published glucose levels of 74 mg% (4.4 mmol/L) for *A. mississippiensis*, and 101 mg% (5.6 mmol/L) for *Crocodylus acutus*. Foggin (1987) reported a normal glucose range of 61.1-117.3 mg% (3.4-6.5 mmol/L) for *C. niloticus*, with a normal mean of 81.6 mg% (4.5 mmol/L). The mean glucose level for *C. niloticus* runts was 73.2 mg% (4.1 mmol/L). Glucose levels for two to four year old *Caiman latirostris* (Tourn *et al*, 1994) was reported as 0.8182 g/L (4.5 mmol/L). In comparison to these other crocodilian species readings, the levels recorded for animals 1, 2 and 3 (9.5 mmol/L, 8.7 mmol/L and 7.8 mmol/L respectively) may be high, possibly due to the reasons discussed previously.

Serum electrolytes (potassium, sodium and calcium) may change quickly, especially in cases of shock. Chloride and bicarbonate compete for sodium in maintaining acid-base balance. Recorded levels of calcium, sodium, potassium and chloride were similar in both the healthy and the sick animals tested. The calcium levels reported here for the sick twelve month *C. porosus* were similar to the plasma calcium levels (range 2.47-2.84 mmol/L) recorded by Morpurgo *et al* (1992) for twelve and eighteen month old *C. niloticus*. Magnesium is required for neuromuscular activity and also influences the use of potassium, calcium and protein. Decreased magnesium levels are found in cases of protein malnutrition and malabsorption, consistent with animals 4, 5, and 6. Phosphate function includes the maintenance of acid-base balance. Decreased phosphate levels may be associated with starvation and malabsorption, again consistent with animals 4, 5 and 6. Grigg (1981) reported overall mean plasma electrolyte levels for non-hatchling wild *C. porosus* of sodium 134 mmol/L, potassium 3.8 mmol/L, calcium 2.8 mmol/L, magnesium 1.5 mmol/L, chloride 118

mmol/L, all of which are similar to values determined in this study. Grigg (1981) also reported a bicarbonate level of 16.6 mmol/L, higher than the results for this study and inorganic phosphate level of 1.95 mmol/L, between the values recorded for healthy and sick animal results in this study. Jacobson (1988a) published plasma electrolyte levels (all in mmol/L) in *A. mississippiensis* and *C. acutus* respectively of sodium 141, 149; potassium 3.8, 7.9; calcium 2.6, 3.4; magnesium 1.5, 1.9; chloride 112, 117; and bicarbonate 20, 11.

Copper is required for haemoglobin synthesis and decreased levels (animal 5) are indicative of protein malnutrition. Zinc is required for body growth and metabolism, and plays an important part in enzyme catalytic reactions. Severe zinc deficiency is manifested in part by growth retardation (known as runt syndrome in crocodiles), dermatitis and poor wound healing, conditions present in animals 5 and others on the farm. Insufficient blood samples from animals 4 and 6 were available to test for copper and zinc.

Anion Gap is the measure of the difference between electrolytes (cations sodium and potassium, and anions chloride and bicarbonate) to determine if an acid-base imbalance is present. In the absence of data on normal anion gap for crocodilians no conclusions may be drawn from the differing values between healthy and sick crocodiles in the study. An elevated anion gap is normally indicative of metabolic acidosis, which is a complication of shock. Blood pH and arterial blood gas analysis would also need to be determined to assist in any conclusions.

Jacobson (1988a) published the osmolality level for *A. mississippiensis* as being 284 mOs/L. The same level for *A. mississippiensis* was reported by Frye (1991) from an amalgamation of multiple cited sources. This is comparable to the values recorded for the three sick younger animals in this study. Increased values in the three large healthy animals may be due to dehydration, or may simply be due to their age as osmolality values for juveniles are generally lower than those of adults. Grigg (1981), however, reported an overall mean osmolality for non-hatchling wild *C. porosus* of 304 mOs/L, somewhat lower than recorded for the three year olds in this study. Osmolality is a very variable measurement in humans with a large normal range, and this may be the case in crocodilians.

It would be unwise to draw any definite conclusions from the biochemical and haematological data presented here. The sample size of three known sick crocodiles and three healthy crocodiles is too small to be reliable. Also, the age difference between the two groups complicates the issue, as may their sex. Animals 1, 2 and 3 were presumed to be healthy as determined by visual observations only. Animals were selected which were unblemished externally, were eating normally and exhibited the normal juvenile behaviour. This may be erroneous as some apparently healthy animals were on occasions found dead within twelve to twenty four hours. At post-mortem some were found to have internal fungal infections.

Additionally, the method of sampling probably affected many of the blood parameters. The animals were captured, restrained and transported by vehicle from the farm to the sampling site adjacent to pathology laboratory A. This was over a distance of approximately forty kilometres with several kilometres of rough dirt road, and at a time delay of at least an hour. Elsey *et al* (1990b) recommended a time delay of a maximum of 10 minutes between initial disturbance of the animal and blood letting to avoid changes to blood biochemistry. Since avoidance of stress in sampling is very important, this could be achieved in future studies by taking sampling equipment and personnel to the animals on site at the crocodile farm, thus avoiding the trauma of restraint and transport of the animals. With trained personnel and ready access to the crocodiles, sampling time could easily be reduced to less than 10 minutes.

Overall, high stress levels may be indicated by raised CPK in animals 1 and 6, and low serum CO_2 in animals 1 and 2. Low albumin, alkaline phosphatase, glucose, magnesium, phosphate, copper and zinc levels, as reported for animals 4, 5 and 6 are consistent with malnutrition and wasting, conditions which were present in those three animals. Low globulin, RBC and amylase levels, particularly in animal 5 are indicative of liver disease, a condition found at post-mortem in animals from the same group with similar symptoms.

Hypoglycaemia or low blood glucose is normally a symptom of stress. Animals with clinical manifestations of muscle tremors, loss of righting reflex, and torpor (Wallach *et al*, 1967, Frye, 1991) were frequently found in a state of catatonia and opisthotonos in the water in their pens and were in danger of drowning. Those animals with

physical symptoms similar to those described by Wallach *et al* (1967) were selected for blood sampling to obtain a biochemical confirmation of the visual diagnosis.

Hypoglycaemia was described in *A. mississippiensis* by Wallach *et al* (1967) and Marcus (1981) and had also been observed in Caimans and False Gavials by Wallach *et al* (1967). The alligator, both in captive and wild states, has a seasonal physiologic variation in blood glucose ranging from 50 mg/dL (2.8 mmol/L) in late Autumn to 100 mg/dL (5.6 mmol/L) in summer (Coulson and Hernandez, 1964 cited in Wallach *et al*, 1967), with hypoglycaemic shock most likely occurring in winter and spring when the blood glucose is at its lowest levels. The blood glucose levels for the three large yearling *C. porosus* in this study (5.2-5.4 mmol/L) correspond closely to the summer levels for *A. mississippiensis.* These readings were obtained in August, a winter month, however all juvenile crocodiles were housed in a heated environment throughout the year and the results may therefore indicate normal summer levels. Glucose levels for animals in a previous study were in the range 7.8-9.5 mmol/L for healthy three year old crocodiles and 3.9-7.3 mmol/L for sick twelve month old crocodiles. The animals in that study had also been housed in a heated environment.

Wallach *et al* (1967) described the usual signs of hypoglycaemic shock in crocodilians as including mydriasis, 'star-gazing', swimming in circles, opisthotonos, torticollis, and catatonic seizures. If untreated in alligators it was normally fatal. It may be induced by stress, which occurred when the animals were being handled or being relocated, and by over-crowding in pens or by being housed with other alligators of different sizes. Competition for food also may cause elevated stress levels. Wallach *et al* (1967) recommended treatment with glucose solutions given parenterally or by stomach tube at a dosage of 3.3 g glucose per kg body weight. Clinical signs in alligators similar to the spontaneously occurring cases were induced by intramuscular injections of insulin (1000 units/kg body weight) with severe hypoglycaemic shock occurring within twenty four hours. Specific diagnosis of hypoglycaemia was made by appropriate laboratory tests and by response to therapy (Wallach *et al*, 1967).

The very similar glucose levels for all three *C. porosus* in this study (animal 1, 5.3 mmol/L; animal 2, 5.4 mmol/L; animal 3, 5.2 mmol/L) are not unusual given that animal 1 had been treated with glucose after the initial blood sampling and prior to the second sample being taken. Animal 1 was manifesting severe symptoms similar to

those described by Wallach *et al* (1967) up to the time of blood letting whereas animals 2 and 3 were normal healthy crocodiles with no prior history of hypoglycaemic symptoms. All of the blood samples had initially been taken at 8 am on the farm in order to minimise disturbance to the animals, and the samples presented to the veterinary laboratory at 10.15 am. When the blood sample for animal 1 was later found to be unusable due to coagulation, the farm staff were contacted by the veterinary laboratory and that crocodile was recaptured and again restrained. It was then transported by vehicle to the veterinary laboratory (a distance of approximately forty kilometres) for further blood sampling with the significance of the intervening glucose dosage not relayed due to communication misunderstanding.

The results for the majority of the blood plasma biochemical tests performed are similar for all three animals with the exception of CPK, GGT, GLDH and AST. Compared with previous results recorded from blood serum from another six crocodiles, globulin levels for these three animals are lower, as are bilirubin, creatinine, urea and magnesium levels. The absence of data on blood biochemistry for the various age groups of *C. porosus* precludes determination as to which group of animals has displayed normal levels. Calcium levels were not able to be tested due to the unsuitability of oxalate preserved plasma.

High CPK activity is indicative of muscle damage but is also associated with stress. The medium to high level recorded for animal 2 may have been induced by the sampling technique and the high level for animal 1 was most likely directly related to the stress of two bouts of handling and restraint, coupled with vehicle transportation. High GLDH levels indicate liver cell lysis with most animal species having an acceptable range of 0-20 IU/L. The levels of GLDH for these three animals are higher than those determined in a previous study.

Haematological values were similar between animals 1 and 3 except for the leucocyte differential count. More neutrophils and less lymphocytes on a percentage basis were found in animal 1 which was suspected as suffering from hypoglycaemia. This may indicate an underlying anaemia or infection in this animal and a high level of stress. Canfield (1985) reports much lower lymphocyte values of 0.6-9% in *C. porosus* blood. However, the four animals in that study were housed in a controlled artificial environment and, therefore, may have been under more stress.

Animal 1, as well as those previously treated for hypoglycaemia on campus, and others on the farm with similar symptoms, responded well to the dosage of glucose. There was an immediately perceptible relaxation of the animals' body and leg muscles, a return of normal pupillary function, cessation of 'star gazing' and body tremors and a return to normal movement from any paralysis. Treatment was usually repeated daily for three consecutive days with relapses only occurring if further major stresses affected the animals soon after the previous treatment. Most appeared to recover fully with resumed appetite and growth. Coulson and Hernandez (1964) cited in Wallach *et al* (1967) stated that alligators are hungry only when they have high glucose levels (in summer) and that alligators subjected to social stresses had low liver glycogen values. This may also true for *C. porosus*. The low glucose levels may reduce their interest in feeding, resulting in a loss of body condition. Repeated adrenergic stimulation and response brought on by continued stresses may further deplete liver glycogen and, combined with lowered body resistance related to poor condition, may precipitate hypoglycaemic shock.

At the commencement of this research program one of the aims was to describe the histopathology of the infection. This aspect was reduced to recording histological presence of fungal elements with identification and confirmation through the mycological studies. Assistance was rendered by staff at a Veterinary Pathology Laboratory who processed histological material and some mycological samples in times of high hatchling and/or yearling mortality (Pierce and Hill, 1992, 1993). Detailed descriptions of the veterinary histopathology was determined as being more relevant to those with more experience in that field.

An ongoing problem for histological preparations was the suitability of crocodile post-mortem tissue. The pens were checked regularly for carcasses during the day and before departure by staff. However, deaths of juvenile crocodiles frequently occurred during the night and because of the high temperatures maintained in the indoor pens, tissue autolysis had often already commenced by the first check at 6 am. This tissue was still suitable for mycological studies. By using the ring method of Harrower (1989a) surface contaminating bacteria on the tissue samples were able to be separated from the slower growing fungi. Bacteria were confined to the surface of the agar inside the ring whereas the fungal hyphae penetrated through the agar and under

the aluminium ring to result in contaminant free cultures outside the ring (refer Plate 12b, section 3.3)

Because of characteristic coloration, some fungi *eg. Cladosporium* sp. may be viewed histologically using standard staining techniques (haematoxylin and eosin, Brown, 1978). Other fungi may only be seen in histological sections after the use of special stains, one of which is the PAS reaction (Brown, 1978). Also, although the presence of fungal elements may be demonstrated in tissue when viewed histologically, this alone does not identify the species.

With reference to Webster (1970), Rippon (1988) and Barnett and Hunter (1987) the classification of the fungi isolated in this study are as follows:-

Kingdom	Mycota
Division	Eumycota
Sub-Division	Deuteromycotina (often referred to as the Fungi Imperfecti)
Class	Hyphomycetes

Aspergillus sp., *P. lilacinus*, *Cladosporium* sp. and *F. solani* are all of this classification to class level. *F. solani* (Mart.) Sacc. also has a teleomorph stage known as *Nectria haematococca* Berk and Broome.

The Deuteromycotina include the majority of medically important fungi causing three types of disease, superficial or cutaneous mycoses, subcutaneous mycoses and systemic mycoses.

All of the fungi which were frequently isolated in this study have septate hyphae thus identification by histopathology alone is not possible. The hyphae of *Cladosporium* sp. are dematiaceous, but the hyphae of *Aspergillus* sp., *Paecilomyces* sp. and *Fusarium* sp. are all hyaline. Mycological culture on various media, with the production of spores, is necessary for complete identification of fungal species.

At the start of this study duplicate cultures were incubated at 30°C and at 37°C. As these fungal species are all monomorphic, later cultures were incubated only at 30°C which is closest to the body temperature of hatchlings crocodiles kept in a 32°C environment. Chloramphenicol was added to the growth media to prevent overgrowth of the fungal culture by surface contaminating bacteria from the post-mortem tissue sample (Booth, 1971b, Buckley, 1971, Koneman *et al*, 1978). Chloramphenicol usage is more advantageous than other antibiotics, as the media can be autoclaved after the

addition of the antimicrobial without reducing its activity (Booth, 1971b, Buckley, 1971, Koneman *et al*, 1978). Bacteria grow very quickly, frequently within twenty four hours of the initial culture, whereas fungi grow more slowly so that erroneous results may be recorded when bacteria over-run the colony before the fungus has established. The addition of the antibiotic prevents this. Due to their sometimes slow growth, fungal cultures were incubated for a minimum of three weeks before nil growth was recorded (Collins and Lyne, 1976).

Of twelve anti-fungal agents used for sensitivity tests to ascertain possible usage in the therapy of diseased crocodile hatchlings, fungi in this study proved sensitive to only five - econazole, griseofulvin, ketoconazole, tioconazole, and Tecto® (Lord, 1990). Griseofulvin is only used to treat superficial fungal infections. Its application as a topical treatment is ineffective as it only becomes fungicidal after prolonged oral administration which permits the incorporation of the antibiotic into the keratin layer of the skin (Volk and Wheeler, 1980), hair follicles and nails (Tortora *et al*, 1989). This antifungal agent was used successfully in the treatment of a pathogenic dermatophyte affecting ostrich chicks (Onderka and Doornenbal, 1992). Tioconazole is also a topical antifungal (Budavari, 1989). As the mycoses in the research reported here were known to be systemic as well as superficial, griseofulvin and tioconazole were, therefore, not considered suitable.

When taken orally, ketoconazole is an effective and possibly less toxic alternative to amphotericin B for many systemic fungal infections, although occasional liver damage may occur (Tortora *et al*, 1989). It also has the advantage of an unusually wide spectrum of activity (Tortora *et al*, 1989). Frye (1991) reported that in reptiles affected with deep mycoses, the antifungal agents ketoconazole and griseofulvin yielded mixed results, although those drugs were effective in superficial mycoses. Frye (1991), however, recommended the consideration of their usage in some deep mycotic infections in animals whose value merited the expense of the drugs and the effort required to administer/deliver them appropriately. In preliminary pharmacokinetic studies of ketoconazole in the Gopher Tortoise (*Gopherus polyphemus*), Page *et al* (1988) cited in Frye (1991) reported that oral doses of 30 mg/kg given once every 32 hours maintained an effective plasma concentration of the drug. Both econazole and ketoconazole are antifungal treatments taken orally and are

available under various trade names (Budavari, 1989). Econazole can be used for systemic infections and topically against dermatophytes (Jones, 1975). These antimicrobials are usually available by prescription only and, therefore, would not be suitable for wide scale treatment of crocodiles.

Thiabendazole is a veterinary anthelmintic taken orally and is used as a systemic fungicide in plants (Budavari, 1989). According to product material (Merck Sharp and Dohme Int., 1977), Tecto® (thiabendazole) is a broad spectrum, systemic fungicide highly effective against a wide range of pathogenic fungi affecting plants. It is non-hazardous to humans and other animals, with its control of plant fungal disease by both protective and curative action. Plant pathogenic fungi controlled by Tecto® (thiabendazole) include eight *Aspergillus* species, *Cladosporium* spp., eight *Fusarium* species including *F. solani*, (Merck Sharp and Dohme Int., 1977) as well as a range of other fungi which have also been reported to affect reptilians. No mention was made of *Paecilomyces* species in that report.

Tecto® is also effective as a surface drench for soil and for the treatment of empty storage areas such as grain silos, greenhouses, and poultry shelters (Merck Sharp and Dohme Int., 1977). Veterinary advice (Turner, pers. comm.) was that a prescription was not required for the supply of thiabendazole and that it was readily available through stock and station agents or animal food barns. Turner (pers. comm.) also advised that the product was non-toxic to animals, was normally dosed as a fluid drench, was made up as a suspension as it is essentially insoluble in water (solubility in water 0.03 mg/ml), and that when applied as a drench thiabendazole was absorbed quickly through the stomach wall with excess released through the urine.

Robinson *et al* (1964) and Fleischmajer *et al* (1965) reported that thiabendazole had fungicidal as well as fungistatic properties against various saprophytic and pathogenic fungi. Robinson *et al* (1964) reported that thiabendazole was a potent, broad spectrum antimycotic agent *in vitro* requiring only low concentrations (μg/ml) for effectiveness. Its stability, lack of taste and colour, low level of toxicity and high plasma levels reached when administered to dogs, suggested to Robinson *et al* (1964) that thiabendazole might be a useful agent in the effective treatment of animal and human mycoses. Robinson *et al* (1964) also reported a range of fungi susceptible to thiabendazole, including *Aspergillus* spp., *Fusarium* spp., and *Paecilomyces* sp. When

used topically thrice daily for an extended period, superficial mycoses in humans were successfully treated (Fleischmajer *et al*, 1965).

Bailey and Jeffrey (1989) reported that thiabendazole had antifungal activity in the same category as formalin against aquatic fungi in artificially infected fish eggs, but that it had toxic effects against the fish eggs which formalin did not. Thiabendazole was used to significantly reduce mould spore counts in litter used in turkey confinement housing (Fate *et al*, 1987). No adverse effects were displayed in the turkeys, when live or in the tissues at post-mortem. Microscopically the lungs of birds housed on untreated litter had a higher incidence of granulomas and mycelia than lungs from birds on thiabendazole treated litter.

Jones (1969) reported the successful treatment of human oculomycosis using thiabendazole in conjunction with other antifungal agents including pimaricin, where previous treatment using nystatin, griseofulvin and amphotericin B had failed. Follow-up *in vitro* tests by Clayton (1969) showed that thiabendazole produced inhibition of *F. solani* but not *A. fumigatus*. Jones (1975) reported that thiabendazole was non-toxic, well absorbed when taken orally and could also be used by topical administration. It was particularly active against *Fusarium* sp., *Penicillium* sp. and *Cladosporium* sp. and was effective in eradicating the fungus *F. solani*. Intra-ocular infections with the causative agent *P. lilacinus* have been cured using thiabendazole (Miller *et al* 1978 cited in Rippon, 1988).

Marcus (1981) reported the use of thiabendazole as an anthelmintic for the effective treatment of ascarids and various intestinal nematodes, including strongyloid nematodes, in captive reptiles. Treatment of strongyloid nematode infection in snakes was given as a fluid drench at 50 mg/kg initially, and with a repeated treatment after two weeks, and further re-dosing every six weeks. Treatment of oxyurids using the same dosage of thiabendazole was also recommended. Frye (1973) reported the use of thiabendazole for treatment of nematode infections in reptilians and Frye (1991) also reported the use of thiabendazole at 50-100 mg/kg repeated fortnightly for the same applications. Frye (1973) also stipulated that as it is a very hygroscopic compound, thiabendazole should be premixed in order that the final dilution will not cause further expansion in volume, which, if this precaution was not observed, could result in fatal gastro-intestinal impaction or fluid and electrolyte shifts in the treated animal.

No reports were found of the use of thiabendazole as an anti-fungal agent in reptilian mycoses. As thiabendazole has had recorded and recommended usage as an anthelminthic in reptilian species it was assumed that it would not cause side effects or toxicity problems in crocodiles. A dose rate of 50 mg/kg was selected based on sensitivity test results (Lord, 1990) and literature review. No tests to confirm effective plasma concentration of thiabendazole in the treated crocodiles were carried out during the course of the treatment.

Jacobson (1978) reported that at that time no treatments had been recorded for mycotic infections in snakes, as most cases were diagnosed post-mortem. Because many of the mycotic and actinomycotic organisms induce granulomatous inflammatory responses in reptiles, antifungal chemotherapy may not prove effective (Frye, 1991). Absorption or migration of effective concentrations of antifungal agents into the lesions is often impeded by encapsulations which form around these granulomata (Frye, 1991). This was noted for future reference if treatment proved to be unsuccessful in the crocodiles.

Although *F. solani* and *Cladosporium* sp. were sensitive to Virkon S®, (refer section 3.3.1), *P. lilacinus* and *Aspergillus* sp. were not. Therefore, this product was not suitable for use as a disinfectant. However, others have reported successful applications against *Fusarium* sp. (Antec International Ltd, 1991), and a pathogenic dermatophyte, possibly *Trichophyton* sp. (Onderka and Doornenbal, 1992).

Malachite green has commonly been used for many years as a topical fungicide, parasiticide and anti-protozoal agent in fish farming by bath or flush methods (Budavari, 1989). Its principal use has been to prevent overgrowth of oomycetous fungi on incubating fish eggs but it has also been used extensively to treat adult fish (Gerundo *et al,* 1991). In a preliminary study of the pathological effects of repeated doses of malachite green, Gerundo *et al* (1991) subjected healthy rainbow trout to a series of exposures to 1.6 ppm (sub-lethal) malachite green baths for forty minutes at weekly intervals. Their results indicated morphological changes to both liver and gills with impairment of normal function. Although all fungi tested in this study were resistant to malachite green at 5 ppm, this product has been used by others for the treatment of fungal disease in *A. mississippiensis* (Jacobson, 1981), *C. niloticus*, (Foggin, pers. comm.) and turtles (Jacobson *et al*, 1980) with, however, a side effect

of mild conjunctivitis in the turtles. Testing of crocodilian species should be carried out to determine any pathological effects similar to those discovered by Gerundo *et al* (1991). Hatai and Egusa (1978) and Hatai *et al* (1978) in their studies of *F. solani* causing black gill disease in cultured Kuruma Prawn, *P. japonicus*, reported that malachite green completely inhibited mycelial growth at 6.3 ppm but allowed growth at lower concentrations. Sodium di-chloro-iso-cyanurate killed the conidia at 6.2 ppm, but not at lower concentrations. All strains of the fungus except two, were resistant to cycloheximide. However, in another study, Singh and Nene (1965) cited in Barron (1971) used Czapek Agar containing malachite green at 50 ppm and another fungicide Captan at 100 ppm as a selective medium for the recovery of *Fusarium* species from natural field soils. The medium, although lethal to conidia and chlamydospores of *Fusarium* species apparently allows the development of *Fusarium* colonies from vegetative hyphae. Therefore, using malachite green as a fungicide against *F. solani* would not be effective.

Fungi isolated in this study were not subjected to sensitivity tests using cycloheximide and sodium di-chloro-iso-cyanurate. This second product is the active ingredient of several brands of detergent/sanitisers registered for use in Queensland, and has been used in the poultry industry for bacterial and fungal decontamination of eggs for human consumption and chick production (Bell, 1986). Based on the results of Hatai and Egusa (1978) and Hatai *et al* (1978) mentioned previously, its use as a fungicide for decontaminating crocodile eggs was rejected. Other products not selected were Hibitane (chlorhexidine) which although relatively non-toxic is inactive against spores; products containing quaternary ammonium compounds or hypochlorites which are both non-sporocidal; and phenolic based products which can be very toxic but are only weakly sporocidal (Collins and Lyne, 1976).

Arocide®, a viricide, fungicide, bactericide and sporocide used as an aqueous solution, was the only product tested which was successful in preventing fungal growth in all species isolated. Arocide® is a readily available and relatively inexpensive veterinary and agricultural product. One of its chemical constituents is glutaraldehyde which is commonly used clinically for the cold sterilisation of instruments which would be adversely affected by autoclaving. Care is required in the use of Arocide® due to the glutaraldehyde content which with repeated skin contact

can cause eye irritation (Budavari, 1989) and irritant and allergic contact dermatitis (Budavari, 1989; Emmett, 1991). Application for usage as an egg disinfectant dip was 1:150 for 5 minutes. No embryonic deaths were attributed to adverse effects of the product with 100% hatch rate of fertile eggs from some clutches. For cleaning and disinfecting sick bays, pens and incubation facilities, more concentrated solutions (up to 1:50) were successfully used according to the manufacturer's recommendations. Although not formerly known to be used for the disinfection of crocodile eggs (Kelso, pers. comm.) Arocide® had been used at 1:150 sprayed in and on farrowing pens in the pig industry, as well as for equine and bovine applications (foaling and calving).

Formalin was reported as a highly effective fungicide against aquatic fungi infecting fish eggs by Bailey and Jeffrey (1989). Formaldehyde gas is a fungicide and formaldehyde solution (formalin) is a germicide and fungicide for plants and vegetables, destroying flies and other insects, and has been used for prevention of mould in stored wheat and grains (Budavari, 1989).

Prior to the commencement of this study, formaldehyde gas (formaldehyde solution mixed with potassium permanganate crystals liberating formaldehyde vapour) had been used at Koorana Crocodile Farm for decontaminating incubated pheasant's eggs (Lever, pers. comm). Its use as a disinfectant in the poultry industry has been long standing and widespread (Wilson and Mauldin, 1990). However, due to current trends in workplace health and safety, and the known health risks to humans (intense irritation to the mucous membranes, irritant contact dermatitis through repeated topical use, and possible carcinogenic properties (Budavari, 1989)), formaldehyde vapour was never used in the work reported here. There was no known previous use of formaldehyde vapour or formalin solutions in disinfection of crocodile eggs and, therefore, of any detrimental effects on developing crocodile embryos.

Other proprietary fungicidal products available could possibly be used in place of Arocide®. However, tests should be carried out to determine which fungal species are sensitive to the products before they are selected for general use. Other factors to take into consideration from the farmer's point of view are:- product availability, ease of application, cost efficiency, and health and safety aspects (for both human and animals).

Prior to the commencement of this research program one of the aims was to have been a survey of crocodile farms in Australia to compare and contrast husbandry methods and environmental conditions with those of Koorana Crocodile Farm. Initial contact with some farmers revealed that this was the basis of another research program which has since been reported elsewhere (Buenviaje, 1991; Buenviaje *et al*, 1994). Therefore that side of the project was discontinued and data was sourced from those reports.

Buenviaje (1991) and Buenviaje *et al* (1994) indicated that winter and low temperatures were implicated as being a predisposing factor in fungal disease of crocodiles at farms situated at the highest latitudes. However, one of the farms cited in their reports was the investigative site for this project and all hatchlings and yearlings were, and currently are, held indoors in a heated environment. Therefore, the initiation of fungal disease by cold stress was less likely here than at farms closer to the equator which do not provide additional heating in the cooler months.

It was found that at initial contact crocodile farmers in Australia were reluctant to discuss disease problems relating to fungi and in some cases indicated that no problems existed. However, when referring to the literature and the relevant researchers the reverse was found to be true (Muir and Cunningham, 1990; Buenviaje, 1991; Melville, 1993; McInerney, pers. comm.). Others indicated that although some problems existed they were not large scale (Onions, pers. comm., Freeman, pers. comm.). An observation by McInerney (pers. comm.) was that for every one crocodile hatchling that had its cause of death diagnosed, many others went undiagnosed and unreported. Carcasses were usually only presented if something appeared 'unusual'. Otherwise many hatchling and embryo deaths were classified under a blanket 'hatchling and egg mortality' description.

On smaller farms where each individual animal assumes a greater significance and monetary value relative to overall numbers, (as is the case at the farm in this study) disease problems become of major importance. A certain number of hatchling and embryo deaths may cause insignificant losses to a large farm with several thousands of hatchlings and a licence to collect eggs from the wild. However, the same number of deaths may constitute a far higher proportion of stock to a small holding, particularly when egg collection from the wild is not permitted by State Legislation and all stock must be bred on the farm.

After informal discussions with other researchers and crocodile farmers it became apparent that the common view held was that the fungal problem as described in this report was because of the latitude of the farm. The assumption was that the colder climate was the cause of hatchling death due to cold stress. However, the geographical location of the farm is within the natural range of *C. porosus* with sightings not uncommon in the local area and nesting known to occur. Also, all crocodiles at the farm and up to two to three years old depending on their size, were housed indoors in heated and insulated pens. At the commencement of this study, ambient air heating was provided day and night in the colder months by heater fans, and during the night only occasionally in summer. Heating is now also provided routinely overnight even in summer months. The water in the pens was not heated prior to this study. After data logger monitoring of the water temperature in several pens, an in-line gas heater was connected to the water supply. This eliminated the possibility of temperature shock previously experienced when the pens were drained and refilled. Water temperature in the pens has since been maintained at approximately 32°C, the same as that suggested by Foggin (1988) for *C. niloticus*. Studies on water temperature and its relationship to stress in hatchling *C. porosus* were in progress concurrently (Turton, 1993) with this research project. Early results from that project suggested that high water temperature (36°C) was more stressful to hatchlings than low temperature (28°C), with the optimum being 32°C (Turton *et al*, 1994). Clutch of origin was also having marked effects on hatchling growth in those trials.

The applied research attempted in the research project reported here was subject to all the inherent problems associated with a working farm which occur from time to time. During the course of the research changes in farm staff have occurred. Fortunately the hatchery/incubation officer has been a long term employee and therefore the small animals are not subjected to the stress of different procedures. During the course of the research several natural disasters affected the district (*eg.* major floods, a cyclone, bush-fires), and, being in a rural area, storms and lightning strikes frequently brought down trees over power lines with subsequent power cuts to the farm. Auxiliary generator power is now available to cater for power losses and so help prevent hatchling deaths due to cold stress. It is acknowledged that regular exposure to lower than normal temperature (*eg.* lack of heating overnight) will increase the hatchlings'

susceptibility to disease (Foggin, 1988). However, a sudden failure of all heating equipment was sufficient on one occasion to cause the deaths of approximately forty apparently healthy hatchlings with mortality commencing within two days of the heating malfunction, peaking at five days and ceasing within the week. Post-mortems, including the usual mycological investigations, could find no clinical reason for death (Pierce and Hill, 1992).

Frye (1991) suggested that the incidence of mycotic infections of a superficial nature in aquatic or semi-aquatic reptiles such as crocodilians "may be directly or indirectly related in the pH of the water because the growth of most fungi is inhibited at hydrogen ion concentrations of less than a pH of 6.5". However, Hatai and Egusa (1978) were able to culture *Fusarium* sp. in broth at a pH range of from four to eleven. Altering the pH of the water in the pens to outside this range would not be a viable option.

Exclusively female *C. porosus* are produced in the incubation temperature range of 28-31°C, and predominantly females at 33-34°C, whereas males are produced in varying proportions around a mid-range peak between 31°C and 33°C (Lang, 1989). Incubation room temperature for this study was set and maintained at 33°C producing an internal egg temperature of 32°C, thus producing predominantly male hatchlings which generally grow faster to reach culling and skinning size sooner (Lever, pers. comm.).

In a clutch of Indian Python eggs, *F. oxysporum* was reported as growing over the membranes as a white mycelial felt and invading the embryo and yolk-sac (Austwick and Keymer, 1981). The same authors reported the isolation of *Chaetomium globosum* from the membrane of hatching boipevussu snake eggs and made the observation that fungal infection in reptilian eggs is probably a factor in hatchability at least in captive animals. Patterson (1974) and Branch and Patterson (1975) both cited in Austwick and Keymer (1981) also alluded to the possible roles of 'moulds' in mortality and abnormal embryo development in reptiles.

Rebell (1981) and Austwick (1984) reported that marine turtles represented the reptilians most susceptible to *Fusarium* infections with investigations attracting attention at that time because of the commercial development of turtle farming. The commonest infections appeared to be those of newly hatched turtles and their

eggshells. *F. solani* was found in lesions on captive baby Atlantic Ridleys (*L. kempi*) collected from the wild (Rebell, 1981). It was also implicated in the deaths of farmed Green turtles (*C. mydas*) collected from *Fusarium* contaminated sand but incubated and hatched in confinement (Rebell, 1981), and also in the deaths of farmed Loggerhead turtle hatchlings (*C. caretta)*, with the eggs hatching in contaminated sand but later reared in confinement (Rebell *et al,* 1971; Rebell, 1981). Horse-hair matting used as a sea water filter at one facility was found to be contaminated with *Fusarium* (Rebell, 1981). *Fusarium* sp. was also isolated from wild Loggerhead turtle eggs with high mortality (Gyuris, pers. comm.) with the nesting sand also thought to be one of the contributing factors.

Foggin (pers. comm.) believed contaminated nesting material was associated with neonatal mycotic skin infections which caused high mortality in *C. niloticus*. Malachite green (1-2 g per 2000 L) was used as treatment. A contaminated terrarium in which an adult snake had lived was suggested as the most probable source of fungal infection in snake eggs with the causal agent *F. solani* (Kunert *et al,* 1993).

Since fungi are ubiquitous in the farm environment attempts to eradicate them would be impracticable. Natural nests are constructed by the crocodiles in areas adjacent to three large lakes and several smaller pondages (refer Plate 2a and Section 1.3). The size of the farm precludes the use of saturation surface drenches for the soil. Tecto® has been recommended and promoted for this application (Merck Sharp and Dohme Int., 1977). Soil solarisation is a process whereby the heat of the sun through plastic sheeting raises the underlying temperature of moist top soil and sub-soil to temperatures which are lethal to many soil bacteria and fungi (DeVay, 1990a, 1990b). The process depends on soil structure, soil moisture, intensity and duration of sunlight, and the composition of the plastic sheet (DeVay, 1990a, 1990b). Not all organisms are killed but they may be weakened sufficiently that they become vulnerable to biocontrol by other organisms, eg *F. solani* controlled by antagonistic G+ve bacteria (Kaewruang *et al*, 1989 in DeVay, 1990b). Clearly, treating the whole farm by soil solarisation or by fungicidal surface drench would not be viable. Treating small selected nesting areas would also not be practicable as the female crocodile constructs her nest by selecting a site suitable to her, not to the farmer's convenience. Disturbance of the female or nest may result in her selecting another site and building

again, or it may sometimes cause her to lay eggs on the bare ground or in the water, therefore resulting in high embryo mortality from physical damage, desiccation and overheating (Lever, pers. comm).

Crisp and Bland (1990) reported on the potential use of ozone as a disinfectant for sea water in the control of pathogenic fungi causing diseases of cultured marine crustaceans. Ozone was found to be effective against *F. solani* with 100% spore mortality at between six and nine minutes treatment. However, the magnitude and cost of such an operation in the crocodile farm situation precludes further contemplation.

Davis *et al* (1986) reported that fungal contamination of poultry eggs in North Queensland was sporadic but persistent and although it occurred mainly in the hot humid monsoon season, fungal growth was also reported in the cool dry winter months. The poultry egg has mechanisms which protect it from both fungal and bacterial invasion and these include the cuticle, shell, shell membranes, lysozyme, conalbumin, avidin and pH of the albumen (Davis *et al,* 1986). Freshly laid eggs are normally sterile internally but the shells become contaminated from the time of laying (Davis *et al,* 1986). Fungi and bacteria from various sources can grow through a moistened shell and into the egg's interior and another expedient route is through cracks in the shell (Davis *et al,* 1986). Although no fungi were isolated from the interior of eggs surveyed by Davis *et al* (1986) a wide range was reported as isolated from the shells. These included *Aspergillus* spp., *Cladosporium* spp., *Fusarium* spp., and *Paecilomyces* spp., all of which were isolated in the crocodile research presented here. Also isolated from poultry eggs by Davis *et al* (1986) were other fungi which have also been reported as causal agents in fungal disease in crocodilians, *eg. Cephalosporium* spp. (Trevino, 1972), *Mucor* spp. (Jasmin and Baucom, 1967), *Penicillium* spp. and *Curvularia* spp. (Huchzermeyer and Agnagna, 1994; Muir and Cunningham, 1990; Muir, pers. comm.) and *Nigrospora* spp. (Muir and Cunningham, 1990; Muir, pers. comm.).

According to Manolis and Webb (1991) fungi were not a factor causing embryonic death of *C. porosus* during incubation but usually only appeared on eggs that were already dead. Eggs that were collected by their research team in the Northern Territory were transported in nesting material and then washed without detergent under tap water at about 25°C. A light scourer was used to remove any dirt and vegetation

adhering to the egg. *C. porosus* eggs were also routinely washed before incubation at a large crocodile farm in North Queensland (Onions, pers. comm.). Manolis and Webb (1991) found that washing did not affect hatchability which was similar to the results of Joanen and McNease (1977) cited in Manolis and Webb (1991) who found no 'appreciable' differences in hatchability between alligator eggs washed with mild dish washing detergent and those left unwashed. Joanen and McNease (1977) cited in Manolis and Webb (1991) also treated alligator egg nesting media with a 2 ppm solution of copper sulphate as a precautionary measure to reduce the growth of 'undesirable fungi' but they did not indicate whether fungi were implicated in embryonic mortality.

Schumacher and Cardeilhac (1990) isolated *Fusarium oxysporum* from both wild (Louisiana) and farm bred (Florida) *A. mississippiensis* eggs with additional fungal species *Penicillium fellucanum, Paecilomyces aviotti* and *Aspergillus niger* isolated from two of those eggs. Fungal hyphae were demonstrated in the egg membranes and in the chorioallantoic membranes. Prior to incubation each egg had been marked and disinfected with a 1% Nolvasan solution (a proprietary brand topical antibacterial, antiseptic disinfectant with chlorhexidine base. Chlorhexidine is inactive against spores (Collins and Lyne, 1976; Tortora *et al*, 1989)). Inoculation of disinfected, uninfected infertile eggs with pure suspensions of the fungal species resulted in only *F. oxysporum* creating lesions on the egg membrane similar to those found in naturally infected eggs. Grossly affected eggs showed retarded embryonic development and embryonic death. Surviving embryos were smaller at hatching compared with hatchlings from uninfected eggs. The route of invasion of the eggs by fungal elements was not determined by Schumacher and Cardeilhac (1990) and they speculated that fungal infections in the female alligator reproductive tract may have been a source of initial egg infection. The fungus contaminated soil in alligator nests was also suggested as a possible source of infection. It is interesting to note that this study by Schumacher and Cardeilhac (1990) was being carried out concurrently but without the knowledge of this author while this study of *C. porosus* eggs was in progress and that similar results and observations were made. A *Fusarium* sp. was identified as the causal agent of disease in both groups of eggs and both authors found

the fungal pathogen ubiquitous in the environment. Surviving embryos in both studies (*ie. A. mississippiensis* and *C. porosus*) were smaller at hatch.

During the egg cleaning and dipping process used in this study, extra care was needed as mechanical stress may cause damage or death to the embryo. During the first season of washing and dipping, some of the embryo deaths could be attributed to mechanical stress on the eggs. This may be caused by the tearing away of the attachments of the developing embryo to the inner upper surface of the shell membrane (Manolis and Webb, 1991). Movement-induced mortality in eggs of the Green Sea Turtle *Chelonia mydas* has also been caused by disruption of the egg membranes (Parmenter, 1980).

Bell (1986) in a report on poultry egg sanitising procedures for both the edible egg market and for chick production, outlined recommended procedures involved and listed suitable detergent sanitisers, all of which had the active ingredient di-chloro-iso-cyanurate. The reduction or elimination of heavy bacterial and fungal contamination from the surface of the poultry eggs avoided poor hatchability and chick quality (Bell, 1986). However, one of the major differences between crocodilian and poultry eggs is that the poultry egg is rolled regularly during incubation and handling of the chicken egg does not pose the problem which it does for the crocodile egg.

Unfavourable environmental temperature between oviposition and collection directly effected chicken eggs with resultant thinning of the albumen or weakening of the egg shells (Sauveur and Picard, 1987). Shell quality of chicken eggs has a direct bearing on the ability of bacteria to penetrate into the interior of eggs and the resulting infection can have severe effects on hatchability and neonate viability (Spackman, 1987). Eggs contaminated with bacteria produced chicks which weighed significantly less both at hatching and after eighty days of growth (Spackman, 1987). Egg shell formation is dependent on the dietary availability of calcium, thus some effect of nutrition on the matrix of the shell is to be expected (Hurwitz, 1987). Eggs produced by some crocodiles in this study were observed to have very poor shell quality with either thin or granular surfaced shells. This indicates either an oviducal abnormality at the site of shell deposition or a lack of available body calcium in the female. Addition of calcium (in the form of shell grit) to the diet of one breeding female in the lead up to the breeding season prevented the production of 'faulty' eggshells which had

occurred in previous years. Dietary calcium supplements for all breeding female crocodiles before each breeding season are recommended to build up calcium reserves in their bodies, and also after laying to replenish those newly depleted calcium levels. 'Pimpling' on the surface of chicken eggs may be caused by the retention of the egg for a period after it should have been laid and this retention may be due to the imposition of stress or an external disturbance upon the hens (Tullett, 1987). A similar effect was seen in crocodile eggs with the stress perhaps caused by competition for, or delayed selection of nesting site by the laying female crocodile. This stress also often resulted in damaged eggs with cracked or dented shells. If the shell is cracked or broken, the egg is instantly exposed to microfloral invasion (Romanoff and Romanoff, 1949).

The outer surface of the shell is covered by an organic layer of cuticular material (Tullett, 1987). This cuticle bridges and plugs the pores thus preventing microbes from gaining entry to the eggs contents. Hinton (1968) in Oosterwoud (1987) advised against storage of washed chicken eggs because of the removal of the cuticle, particularly if the eggs were dry cleaned with abrasive material. However, Simons and Wiertz (1966) in Oosterwoud (1987) showed that 'proper wet cleaning' did not remove the cuticle. Oosterwoud (1987) opined that wet cleaning and sanitising of eggs, resulting in the removal of dirt and reduction in the number of micro-organisms, might be a measure in the prevention of 'mould' growth. Nascimento *et al* (1992) found that the cuticular layer in chicken eggs was rarely present as an even covering over the shell surface and the paired shell membranes were invariably pitted with holes larger than bacterial dimensions. The occurrence of pitted areas in the shell had a strong effect in damaging the eggshell's capacity to restrain microbial entry (Nascimento *et al*, 1992).

The interior of the newly laid avian egg is usually free of micro-organisms because of the natural protection provided by the physical structure and by the chemical constitution of the albumen (Romanoff and Romanoff, 1949). Shortly after laying, the egg is contaminated by a range of micro-organisms with the species of bacteria or fungi varying according to environmental circumstances. Chicken eggs produced in free range conditions or in deep litter showed a higher degree of internal contamination on yolk and inner shell membranes by bacteria due to the dirtiness of

the egg shell (Sauveur and Picard, 1987). The majority of microbes found on the surface of the avian egg are air, soil or water saprophytes. Moulds as well as bacteria have been demonstrated in the interior of fresh laid avian eggs and may constitute up to 4% of total flora (Romanoff and Romanoff, 1949). *Aspergillus*, *Penicillium*, *Mucor, Cephalosporium* and *Fusarium* spp. were commonly found.

The mode of contamination of the egg may be congenital *ie.* contamination occurs during the egg formation in the ovary or oviduct, or extragenital *ie.* after the egg has been laid (Romanoff and Romanoff, 1949). "Potentially, the pores of the shell are avenues for the entrance of bacteria and fungi into the egg's interior" (Romanoff and Romanoff, 1949). As early as 1851, von Wittich cited in Romanoff and Romanoff (1949) demonstrated the ability of moulds to enter the avian egg through the shell and experimental evidence suggested that fungal hyphae enlarged the pores of eggshells thus facilitating the entry of bacteria (Zagaevsky and Lutikova, 1944 cited in Romanoff and Romanoff, 1949). The egg is protected from micro-organisms by firstly the cuticle and the shell, secondly the shell membrane, and thirdly by the albumen. The albumen contains a germicidal substance, lysozyme, which completely lyses many organisms. However, not all microbes are equally susceptible to the lytic power of egg albumen.

Micro-organisms remain at the surface of the shell unless some agency promotes their translocation through the pores (Board and Fuller, 1974). Three methods have been recognised:- (a) infiltration of the pores by fungal hyphae, (b) flooding of the pores with water drawn in by capillary attraction (and hence passive translocation of micro-organisms) and (c) the sucking in of contamination when a warm egg contracts on cooling (Board and Fuller, 1974).

Fungi are able to use the nutrients on the shells surface until that supply is exhausted and are then able to penetrate through the shell and multiply within the egg (Romanoff and Romanoff, 1949). Initial growth may be confined to the inner surface of the shell, the outer surface of the membrane or to the air cell. The membrane probably offers no barrier at all to the infiltrating hyphae of moulds (Board and Fuller, 1974). Most fungi find a favourable environment within the egg and may penetrate the albumen where they often cause coagulation of the areas surrounding their growth (Romanoff and

Romanoff, 1949). If the fungi reach the yolk, they may cause rupture of the vitelline membrane. The coagulation effect had been seen in the crocodile eggs in this study.

The chick embryo increases resistance to infection as it develops (Board and Fuller, 1974). This may also be true in crocodilian embryos as infection in freshly laid eggs in the nest was common but experimental inoculation in much older embryos failed to cause infection. The dipping of warm eggs in chilled suspensions of bacteria has been used to experimentally infect contents of domestic hens eggs. However there was a lag of upwards of 10 to 15 days between initial challenge of the eggs and onset of infection (Board and Fuller, 1974). This may also be true for the crocodile eggs in this study which hatched within a few days of being inoculated with fungal conidia and thus had insufficient time to incubate infection.

Ferguson (1981) and Wink *at al* (1990) in studies of alligator eggshells using scanning electron microscopy, both reported the presence of numerous micro-organisms around the surface defects and erosion craters in the pores. These microbes were found to be necessary for the extrinsic microbial degradation of the alligator eggshell. This degradation gradually increased the porosity and decreased the strength of the shell thus allowing emergence of the alligator hatchling from the egg when fully developed. This microbial weakening of the eggshell has not been found necessary in *C. porosus* eggs (Manolis *et al*, 1987). No mention was made by Ferguson (1981) or Wink *at al* (1990) of any internal egg contamination effects on developing embryos.

Solomon and Tippett (1987) carried out studies on intra-clutch localisation of fungal hyphae in eggshells of the leatherback turtle (*Dermochelys coriacea*). The site of nest location was influential upon the incidence of fungi and greater fungal accumulation occurred in the middle of the nest. Turtle eggs with calcitic, as opposed to aragonitic, shells were found to be more susceptible to fungal invasion due to the more open framework of the calcitic shell (Solomon and Baird, 1980 in Solomon and Collins, 1986; Solomon and Tippett, 1987). The normal eggshells of the Nile crocodile (*C. niloticus*) and the alligator (*A. mississippiensis*) are both of a calcite structure, with multi-layers permeated by pores (Solomon and Collins, 1986). In scanning electron microscopy studies of *C. porosus* eggshells during this research, a similar calcite structure was found with the pores appearing as eroded stepped craters (refer Plates

18a and 18b). Fungal hyphae were observed growing between the calcite layers (refer plate 20a) and fungal conidia were seen on the surface of the shell and in the pore crater (refer plate 18a and 19b).

The large eggshell pore size of *C. porosus*, from 100 μm to 500 μm diameter, would readily allow passage of the very much smaller fungal conidia and hyphae through to the inner shell membrane (refer Plates 18a and 18b). *Aspergillus* sp. and *F. solani* were identified from mycological culture prior to scanning electron microscopy studies. The conidia seen in Plate 18b are similar in shape and size to those of *Cladosporium* sp. and *F. solani*. The conidiophore and phialides seen in Plate 19b are similar in structure to those of *Aspergillus* sp. An unidentified species of mite with body width of less than 100 μm was able to gain entry through the pores and cracks of the shell (refer Plate 20b). These mites may act as a carrier for fungal pathogens, thus contributing to fungal contamination of the eggs and, therefore, to embryo mortality. Hyphae were seen on the inner side the shell surface and on the shell inner membrane. Using light microscopy, hyphae had also been seen within the airspace between the shell and membrane (refer Plates 13b). Hyphae were also seen in the airspaces in unhatched eggs (refer Plate 14a).

Wyneken *et al* (1988) in an investigation of reasons for egg failure in natural and relocated nests of the Loggerhead Turtle (*C. caretta)* reported fungal presence in eggs. Several embryos dissected from non-viable eggs showed extensive fungal invasion of possibly *Mucor* sp. However, the fungal growth could not be associated with a particular category of nest location, *ie.* whether natural nest, reburied nest or incubator nest. Fungal invasion has been implicated as a reason for turtle embryo mortality in two other cases. Solomon and Baird (1979) cited in Wyneken *et al* (1988) had described the invasion of *Aspergillus* sp. into the shell membranes of eggs of the Green Sea Turtle (*C. mydas)*, and noted the potentially deleterious effects on the embryos. *F. solani* had also been implicated as the causal organism in failure of eggs of Kemp's Ridley (*Lepidochelys kempi*) which had been incubated in polystyrene containers (G. Leong, pers. comm. to Wyneken *et al,* 1988).

Kunert *et al* (1993) reported the invasion of *F. solani* in fertile eggs of the snake *Elaphe guttata* causing malnutrition in the ophidian embryo. Three of four fungally

infected eggs did not hatch but an abnormally small snake was hatched from the fourth. Similarly in this crocodile egg study, severely infected eggs (*ie.* those with clearly visible external contamination, normally associated with moderate to severe internal infection) failed to hatch. Crocodile eggs in which the external contamination had been removed, but which were still 'speckled' (frequently associated with light internal fungal infection) usually continued to grow. Hatchlings from these infected eggs were visually smaller than healthy hatchlings which developed from eggs which were free of 'speckling' when collected and which remained clear of external contamination during incubation. These small hatchlings were usually slower to commence feeding and were slower growers and may be associated with the 'runt syndrome' or 'maladaption syndrome' described by other authors (Cowan, 1968; Foggin, 1987; Ladds and Sims, 1990; Frye, 1991; Buenviaje, 1991; Buenviaje *et al*, 1994; Lance 1994). Mortality of hatchlings during their first year was usually highest in the smaller hatchlings which sometimes became anorexic. The success rate of force feeding these animals with liquid or solid food varied with the stress of the extra handling possibly outweighing the nutritional benefits. With perseverance, and by force feeding the animals using liquid food early in their anorexic state, some of those less weak animals recommenced self-feeding. Post-mortems of animals which died without external evidence of fungal infections but which were suffering from a loss of body condition frequently resulted in the diagnosis of internal fungal infection combined with malnutrition.

Kunert *et al* (1993) reported that snake eggs with distinctly defective eggshell were frequently invaded by a number of filamentous fungi and bacteria so that the eggs rapidly became addled. However, in that report the snake eggs infected with *F. solani* did not addle even after 59 days of incubation. This is similar to the results found in this study of crocodile eggs, in which eggs with suspected bacterial infections became addled within a few days whereas those with only fungal infections did not. The contents of the former group of eggs liquefied very quickly, with associated foul odour, and rapid autolysis of the embryo. Bacterial identification was not part of this study. Since bacteria grow and reproduce very quickly, *in vivo* bacterial infections and *in vitro* bacterial cultures may become well established within one to two days. The suggestion that fungal infections in eggs may be secondary to bacterial infections is

possible but not likely. Fungi which are slow growers can easily be over run by bacteria but not generally the reverse. As found in this crocodile study and the study by Kunert *et al* (1993) eggs which are fungally infected do not necessarily later become bacterially contaminated, and therefore, do not addle. Eggs with an embryological age of from seven days through to hatching, which were subsequently found to be fungally infected, still had readily recognisable embryos with firm body structure but some coagulation of surrounding yolk, even when deliberately left incubating for several weeks.

As Kunert *et al* (1993) reported that defective eggshells were implicated in the penetration of fungi, and as this crocodile study also found that cracked or dented eggs frequently showed external and internal evidence of fungal contamination, even after being processed through the decontaminating process using Arocide®, it is suggested that these eggs not be incubated with normal undamaged eggs. Although some embryos had survived the physical trauma which caused the shell damage, and also survived any subsequent fungal infection, there is an increased risk of fungal cross-contamination to other undamaged and uninfected eggs. Similarly 'speckled eggs' are also a possible source of contamination during incubation. Although each damaged or 'speckled' egg is a potential hatchling and is therefore valuable to the farmer, this needs to be balanced against the increased risk to other embryos. Separate incubation facilities or an isolation unit within existing facilities might be appropriate.

Wobeser (1994) stated that disease management can be of three types - prevention (precluding the occurrence in animals where it does not occur), control (reducing the frequency of occurrence or the severity of existing disease) and eradication (total extirpation of the disease from the animal population). In the situation reported here steps taken for the prevention and control of the disease were more effective than attempts at eradication by chemotherapy.

In the disease process reported here, the infective agents are endogenous with a wide range of environmental sources and, therefore, it would be impracticable or impossible to eliminate them. Reduction of the conidial numbers in the hatchlings' immediate environment was possible through disinfection of the housing, and by modification to husbandry methods and environmental factors described elsewhere. The most common method of disease control in domestic animals is by destruction of

the agent within the host by the use of a chemical *eg.* an antibiotic. This has limited application in wild animals (Wobeser, 1994) even when in a captive situation. One disadvantage of attempting to eliminate the agent from the host is that substantial injury may have already occurred prior to treatment (Wobeser, 1994). Additionally, repeated and widespread use of biocides to attack the living infective agent may stimulate acquired resistance in the target organism and reduce the effectiveness of the product. Techniques required for investigation and treatment of disease in wild animals often cause physiologic and behavioural changes that may confound the results (Wobeser, 1994). Similarly, in captive wild animals this is also true. Capture, restraint, handling and specimen collection of crocodilians is known to cause changes in blood biochemistry within 10 minutes (Elsey *et al*, 1990b, and this study). The presence of observers in close proximity is sufficient to alter the behavioural pattern of feeding crocodiles hence exacerbating any loss of body condition and further compromising immunological responses.

Other difficulties exist when trying to treat large numbers of diseased crocodiles, not the least of which is the cost of the additional time involved for staff, the expense of the antibiotic, and the inherent dangers of handling non-domesticated and dangerous captive wild animals. Also, the stress placed on the animal while being handled to enable administration of therapeutic products may cause further deterioration in health thus negating any beneficial effect of treatment.

Frye (1991) and Jacobson (1988b) both recommended an injection site for crocodilians as being one of the posterior occipital venous sinuses, also suitable as venipuncture site. In future studies this would be the preferred site instead of the ventral caudal site as used for the biochemical and haematological aspects in this report. The preferred site for intramuscular injection in crocodilians is the foreleg because a renal portal system drains the hind legs of reptiles (Jacobson, 1978, 1988b; Frye, 1991). Prior to the commencement of this research, after veterinary advice to the operators of the farm and supply of an injectable antibiotic (details not recorded), the antibiotic was administered to the anterior tail musculature (Lever, pers. comm.; Reimers, pers. comm.) The majority of that antibiotic would therefore have been removed by the kidney before any benefits were received by the dosed crocodile.

Additionally, at post-mortem, a hard yellow deposit was observed at the injection site in the muscle and the therapy was not considered beneficial.

Nitrofurazone is described as a topical anti-infective and antimicrobial agent which is only slightly soluble in water (Budavari, 1989). It is used for the treatment of various bacterial, protozoal, trypanosomal and mite infections in both human and veterinary applications (Reynolds, 1982) and has also been used for the control of superficial fungal infections in fish at the CQU Aquaculture Centre at Rockhampton. Nitrofurazone administered orally may cause adverse side effects in humans and in high doses is carcinogenic in rats (Reynolds, 1982). Jacobson *et al* (1980) reported that the use of nitrofurazone was unsuccessful in controlling mucormycosis in hatchling Florida Softshell Turtles (*Trionyx ferox*) with mortality reaching 90%.

From the data (refer section 3.11.1) it can be seen that only one hatchling from each of the groups survived the eight week nitrofurazone trial period. As the method of identification of the animals at that time related to investment group and not to clutch, and as each investment group was made up of one or more clutches, no conclusions can be drawn as to whether mortality was clutch related. The survivor from trial B, although from investment group 6, may or may not have been from the same clutch as the animal from investment group 6 in trial A. Similarly, in trial A the two longest survivors (both investment group 7) also may have been unrelated. In the next and subsequent breeding seasons the identification system was changed to reflect clutches and year of hatching. The erratic and increasing mean weight of each group during the 8 weeks of the trial is a data artefact. Due to the early deaths of weaker and more diseased hatchlings during the trial, the mean weight was raised because the stronger less affected animals were still feeding and growing until they too succumbed. Stress for the treated animals was most likely a major factor in this situation, firstly from the extra daily handling and secondly from additional human presence for observations. An additional cause of stress was undoubtedly the weekly weighing and associated handling of the animals. In later trials weighing and measuring was limited to once every four weeks.

Failure of the nitrofurazone treatment trial may be attributed to several factors. The animals in the trial were all chosen because they had obvious signs of fungal disease. At the start of the trial there may already have been too much internal damage to

major organs therefore making any treatment not viable (Wobeser, 1994). Secondly, the method of treatment (by topical bath) could only take effect if and when the hatchlings moved into the treated water. After daily addition of the nitrofurazone to the water each hatchling was forcibly submerged to obtain an initial dose. They usually then quickly retreated to the nearest 'hide' and remained hidden for the duration of observation. During the periods when not observed, it was presumed that the hatchlings would have returned to the water for at least some of the time, and also that they would have ingested some of the nitrofurazone. Even if this assumption is correct, the volumes ingested would have been small, and probably too small for effect. The third and major contributing factor to the failure of this treatment trial was deduced from the information obtained from Tortora *et al* (1989) in the closing stages of the trial. Tortora *et al* (1989) stated that although nitrofurans were effective fungicides, they did not reach high enough concentrations other than in urine to be useful in systemic chemotherapy and were, therefore, only advantageous (for human use) as topical treatments for skin, eye, and vaginal fungal infections. Therefore, the future use of nitrofurazone would only be effective for the treatment of known superficial mycoses.

The suitability and effect of thiabendazole as a treatment for fungal disease was to have been ascertained through growth trials. Results may be referred to in Table 20 and graphically in Figure 3. It was surprising to note that the untreated Group 3 grew better than the two control Groups 6 and 7. This was the reverse of what had been expected based on information on thiabendazole in the scientific literature and from the sensitivity tests.

The food supplements growth trial was established to determine the effect of additional supplements on growth and to ascertain if animals with only basic rations (meat with added calcium di-phosphate) were suffering from nutritional stress which may have been contributing to disease susceptibility. Results in the food supplements trial may be referred to in Table 20 and Figure 4. Group 1 which received only basic rations grew the best, followed by Group 2 which received additional vitamins in the form of Vita-Stress®. Group 3 which received kangaroo meat, calcium di-phosphate, Vita-Stress® and Enzactiv® grew the least. This was the reverse of what had been expected. Enzactiv® was selected as an additional supplement as it had been used to

promote higher growth rates in crocodiles at a farm in Western Australia (Douglas, pers. comm.).

The stocking density trial was to determine the optimum stocking rate with the least stress on the hatchlings. Results of growth trails with hatchlings housed at different stocking densities are presented in Table 20 and Figure 5. Again, results were not as expected with Group 5 which consisted of animals housed at the highest density (40 per pen = 18.2 animals/m^2 = 0.06 m^2/crocodile) producing the best growth rate. This was followed by Group 4 with least density (10 per pen = 4.5 animals/m^2 = 0.22 m^2/crocodile). The poorest growth rates were from Groups 6 and 7 (both with 20 animals per pen = 9.1 animals/m^2 = 0.11 m^2/crocodile). Zilber *et al* (1992) found that high stocking densities of *C. niloticus* had no effect on growth. However, increased mortality was noted in 4-8 month old hatchlings kept at high density during their first winter. Stocking densities for the trials carried out by Zilber *et al* (1992) ranged from 6 to 15 animals/m^2. In contrast, Elsey *et al* (1990) found that the growth rates of juvenile alligators was inversely proportional to stocking density. Lower stocking density groups gained more weight and showed a greater increase in length than did groups at higher stocking densities. Stocking densities used by Elsey *et al* (1990) were 0.35 m^2/alligator, 0.18 m^2/alligator, 0.12 m^2/alligator and 0.09 m^2/alligator. Results for the trials in the study reported here were expected to be similar to those of Elsey *et al* (1990).

Results for all of the groups 1 to 7 may be referred to in Figure 6. When viewed *in toto* and after further investigation as to the reason for unexpected results it became clear that temperature had an over-riding effect on all groups. The 'hot-room', where the pens for the trials were positioned, had been warmed by a heater fan located approximately 1.6m from the floor to the left of the entrance. The room air was further circulated by a electric fan on the floor at the opposite end of the room. Temperature was initially recorded by a thermohygrograph on a shelf above the fan. After examination of the growth results of all seven groups it was noticed that, when groups were ranked in order of final mean weight, this approximately coincided with the distance of the tiered pens from the floor and in relation to the heater fan. Pens (and groups) 1, 2 and 3 were tiered equidistantly on the left side of the room from top to bottom. Pens 4 to 7 were similarly tiered on the right but with less space between

them and further from the heater fan. The availability of a data logger with four temperature probes allowed continuous temperature recording in various locations throughout the room and this confirmed the suspected temperature gradient from ceiling to floor and from left to right, and lack of air circulation in some areas. Temperature in pen 1 (high on the left side of the room and closest to the heater fan) was approximately 32°C while the temperature in pen 7 (the lowest on the right side of the room) was frequently as low as 26°C. Pen 4 was situated highest on the right but due to its close proximity to the low ceiling suffered from poor air circulation.

High mortality (50%) was recorded in groups 4, 6 and 7. This may be related to temperature stress, as groups 6 and 7 were found to be at the lowest temperature and group 4 had poor air circulation. Mortality in Group 3 was the next highest (40%) also possibly due to temperature stress. Lowest mortality (20%) occurred in the pens 1 and 2 which were found to be at the warmest temperature. Interestingly group 5 at the highest stocking density only recorded a mortality of 27.5%. This group was found to be at a temperature similar to that of groups 1 and 2, and the slightly higher percentage of deaths may have been due to the higher stocking density, similar to the effect seen by Zilber *et al* (1992).

The slight drop in mean weight for groups 3 to 7 in the first four weeks may have been due to the stress of relocation and extra handling of the hatchlings in establishing the trials. However groups 1 and 2 which were processed in the same manner were not affected in that way. Thiabendazole was also initially thought to be negatively affecting growth of those groups. However this theory was discarded because group 3 which was not treated also suffered from a drop in mean weight. The most likely explanation again was the effect of temperature and the position of the various groups in relation to the source of heat.

Deaths of animals in the trials did not appear to be clutch related, however, the size of the samples was too small to be certain of this. Larger groups or replicates of groups could not be used due to the shortage of healthy hatchlings of approximately the same size and age, and to allow for allocation of animals from the same clutch to each group. Availability of pens and separate heated indoor facilities was also at a premium.

No definite conclusions could be drawn from the growth rate data regarding the usefulness of thiabendazole treatment or additional food supplements, or optimum stocking density. However, other hatchlings reared in the A-frame were, and have since been supplied with food supplements as a matter of course. Their body condition and growth rates have improved annually when compared with that of each preceding year (Lever, pers. comm., Reimers, pers. comm.). The stocking density of 40 hatchlings per pen did not appear to be detrimental to growth. Therefore, when necessary, 40 to 50 of the smallest hatchlings have since been housed in those pens, thus effecting a savings in both energy costs and space.

The 'hot-room' used for these trials had been scheduled for replacement with a larger more suitable facility. Demolition was brought forward after the results of growth trials and the over-riding temperature effect were confirmed, with subsequent construction of a purpose built incubation room.

To ascertain whether any growth advantage could be gained by housing the hatchlings in darkness, with perhaps an associated reduction in stress to the animals and, therefore, lowered disease susceptibility, a light/darkness growth trial was established. From the results in Table 21 and Figure 7 it can be seen that there was very little difference in weights between the two groups of animals over the three month period of the trial. With reference to Figure 8, the lengths were even more closely matched. From these results it was tentatively concluded that housing the animals in darkness had no advantage over housing in diffuse sunlight, providing that appropriate heating is supplied. Space requirements precluded a longer trial which may or may not have resulted in greater differences. These results were in contrast to those of Zilber *et al* (1991) who used *C. niloticus* in trials to compare the effect of sunlight, darkness and temperature on growth. In those trials the crocodiles housed in direct sunlight grew the best, followed by those housed in the dark, with both groups supplied with heated water. Crocodiles with indirect light but no heat were ranked third and those in direct sunlight but without heat grew the least. Highest mortality occurred in the third group which had the lowest temperature and no direct sunlight (Zilber *et al,* 1991). In the light/dark study reported here all groups of hatchlings were supplied with both ambient air heating (supplied by heater fans) and gas heated water so that the only difference was the presence or absence of diffuse sunlight. From this study it would

appear then that light levels are not as important for growth and hatchling well-being as is temperature. The deaths of three hatchlings occurred during the trial, one from the group of smaller hatchlings in the dark and two from the group of larger animals in the light. All were from different clutches. Insufficient animals and pens were available to set up replicates of the two 'treatments'.

From Volk and Wheeler (1980) Koch's postulates may be summarised as follows:

(1) the same organism must be found in all cases of a disease,

(2) the organism must be isolated and grown in pure culture from the infected animal,

(3) the organism must reproduce the disease and symptoms when inoculated into a susceptible animal, and

(4) organism must then be then isolated in pure culture from the experimentally infected animal.

Although mostly effective, there are some exceptions to these rules as detailed by Volk and Wheeler (1980). For example, the agents of some bacterial diseases (*eg. Treponema pallidum*, the causal agent of syphilis) have not been grown on artificial media. Neither has *Mycobacterium leprae*, the causal agent of leprosy. Some viral diseases also do not fulfil categories 2, 3, or 4.

To determine factors contributing to a disease outbreak in captive reptiles, certain diagnostic procedure should be followed (Frye, 1991). As reported by Frye (1991) these are (a) collection of epidemiological data, (including husbandry and environmental); (b) observation of macroscopic and microscopic lesions and clinical signs (histology, haematology, biochemistry *etc.*); (c) isolation of fungal agents from body tissue (mycology and sensitivity testing); and (d) reproduction of disease in healthy reptiles inoculated with the isolated agents. "This last criterion is optimum if one wishes to fulfil Koch's Postulates in confirming that a particular agent is responsible for inducing a particular disease. Because of the expense involved and/or the rarity of the animals, this last requirement often is justifiably omitted" (Frye, 1991). The use of appropriate reptilian culture tissue if it was available for inoculation of infective agents, rather than the use of live animals, was suggested by Frye (1991). The crocodiles in this research study were very valuable to the farmer, and considerable numbers had already been lost as a result of disease. Therefore, as it was

not known whether Koch's Postulate could be fulfilled in this instance, only small numbers of eggs and hatchlings were selected for experimental infection.

Hanson (1969) and Wobeser (1994) considered the one agent/one disease model was not adequate for the study of wildlife diseases and that Koch's Postulates were particularly inappropriate for some disease investigations. These were (quoted from Wobeser, 1994) (a) many non-infectious diseases, (b) diseases caused by mixed infections, (c) diseases in which predisposing factors are important, (d) diseases with a carrier state, (e) diseases caused by opportunistic agents that may or may not cause disease when present, and (f) many chronic diseases in which the inciting cause has disappeared before the clinical disease becomes apparent. In the situation reported here categories (b), (c) and (e) are of relevance, and possibly also (a).

Wobeser (1994) cited multiple features of each of the agent, the host and the environment which need to be evaluated when investigating disease of wildlife. Even if only one agent is involved, each of these three components will have a variety of determinants, any or all of which may influence whether or not overt disease will occur (Wobeser, 1994). Extrapolated to the situation under investigation in this study, relevant examples of variables for the agent could be dose, method of exposure and duration of exposure. Host variables may include age, sex, nutritional status, reproductive status, past exposure, immune competence, concurrent disease (Wobeser, 1994), genetic heterogeneity and social structure (Hanson, 1969). Environmental variables include temperature, humidity, population density, and water, air or soil contaminants as well as climate and weather. Wobeser (1994) discussed a 'web of causation' in which multiple factors inter-relate to result in disease and where it may be difficult to classify a single factor as a distinct feature of either the agent, host or environment. Hanson (1969) had previously put forward the concept of host and pathogen populations interacting in a varying environment. One single factor may be a necessary component but may not be individually sufficient to produce disease without the presence of other factors. For establishing causation, and taking into account the multifactorial nature of most diseases, Wobeser (1994) lists a new set of criteria to replace Koch's Postulates. One of these criteria is that "elimination or modification of exposure to the hypothetical cause should decrease the occurrence of the disease". This has been attempted in the situation reported here where changes in

the egg processing and incubation procedure has resulted in a decrease in egg mortality. Also, modifications to husbandry methods and environmental conditions have resulted in a decrease in hatchling mortality.

In other studies other workers have been able to experimentally infect reptiles with fungi. Sinclair and El-Tobshy (1969) injected Louisiana Snapping Turtles, *Chelydra serpentina serpentina* and Red-eared Turtles, *Pseudemys scripta elegans* with fungal suspensions of *G. candidum* previously isolated from both plant and clinical materials (human and tortoise). Sinclair and El-Tobshy (1969) were subsequently able to isolate the fungus from post-mortem tissue and also reported that it retained its pathogenicity to plant tissue. El-Tobshy and Sinclair (1969) cited in Sinclair and El-Tobshy (1969) showed that "plant and animal isolates of G. *candidum* grew in the chorioallantoic membrane of embryonated chicken eggs and incited hyperplasia and hypertrophy, followed by tissue disintegration as a response to infection". Georg *et al* (1962) using isolates from fatal lung infections in giant Aldabra and Galapagos tortoises (*Testudo elephantopus* and *T. gigantea elephantina*), were able to experimentally transmit *B. bassiana* to a box turtle, *Terrapene carolina.*

McAleer (1983) cited in Connole (1990) described 'black shell disease' of the Western Rock Lobster, *Panulirus cygnus* with the causal agent *F. solani.* The lobsters were wild, not cultured which is unusual given that most reported outbreaks in crustaceans occur in farmed or cultured species. Although *F. solani* is not a marine organism it can live in saline conditions of up to 10% NaCl (Hatai *et al*, 1978). McAleer (1983) cited in Connole (1990) was able to fulfil Koch's Postulates by the superficial injection of the fungus into the tail flesh of healthy mature lobsters which resulted 100% mortality within eight days. Using *F. solani* conidia initially isolated from newborn Bonnethead sharks, Muhvich *et al* (1989) experimentally infected channel catfish and subsequently re-isolated the pathogen from muscle near the injection site.

Austwick and Keymer (1981) reported that samples of two fungi originally isolated from crocodiles were used for experimental infection of terrapins. *P. lilacinus* was inoculated either intraperitoneally or subcutaneously into two *Lacerta dugesii* and the fungus was subsequently isolated from tissue 28 days later with hyphae visible in histological preparations. Isolates of *M. anisopliae* were inoculated subcutaneously into *L. dugesii* with the fungus subsequently re-isolated from the liver, lung and

kidney after the death of both individuals. Similar inoculations of the fungus into *Pseudemys elegans* yielded similar results. Attempts at airborne infection of similar terrapins with both fungi were unsuccessful.

The clutch of crocodile eggs selected for experimental infection in this study was assumed to be at age sixty days and approximately 25 days from hatching according to nest collection data. However, the eggs hatched eleven days after experimental infection. When pipping was first noticed it was thought that premature hatching was occurring, precipitated perhaps by the recent handling of the eggs. However, each hatchling left its egg of its own accord and was fully formed with no residual egg yolk protruding from the belly and the abdominal wall almost sealed over. None of the hatchlings showed any external lesions at hatching. An error in recording the collection date was not possible as all information was charted sequentially as it was collected. Clearly the eggs were older than initially thought when collected as the majority of the 44 clutches for that season hatched at around 85 days (± 2 days) of incubation and all were incubated under the same conditions. Throughout incubation that clutch had remained free of fungal contamination and 48 eggs had developed normally from 51 fertile eggs initially laid, with no infertile eggs. The three very early embryonic deaths were attributed to possible mechanical stress due to the handling prior to incubation and no fungal contamination was visible nor fungi isolated from those eggs.

The twelve hatchlings used in the trial to substantiate Koch's postulate were experimentally infected at age three days when the abdominal wall was sealed and self-feeding had commenced (mean length 305 mm, mean weight 63 g, N = 12). All were from the one clutch which had been collected, washed, dipped and incubated in the same manner as other clutches for that breeding season and had developed normally without signs of fungal infections on the eggs. That clutch originally consisted of 65 eggs, with one infertile egg and 64 live hatchlings produced and was, therefore, considered to be of healthy stock.

All of the hatchlings from both of the Koch's trials were similarly housed but in separate groups of three in nally tubs. These tubs were normally suitable for housing very young or very small hatchlings in groups of up to six. However, in the Koch's trials they were quickly found to be inadequate with several of the animals managing

to escape over the sides into adjacent tubs or into the main pens holding non-trial hatchlings from the same and other clutches. As the hatchlings could only be identified by clutch and year (by previously described method of scuting) it was impossible to reallocate the escaped animals to their correct treatment group and the trials were discontinued. However, all animals from the two clutches (both trial and non-trial) were observed over the following months and post-mortems were carried out on a small selection of hatchlings when they reached the ages of 15, 17 and 19 weeks.

The failure of the eggs experimentally infected by injection to succumb to fungal infection, and the embryo to die in the egg was possibly due to too short an incubation time (11 days) for the fungus to grow because of the earlier than expected hatching. Due to their slow growth, fungal cultures must normally be incubated for a minimum of three weeks before nil growth is recorded (Collins and Lyne, 1976). Additionally, the eggs selected were healthy and incubated under ideal conditions without any external stresses (*eg.* high or low temperatures, desiccation, contamination from nesting material) which in the past may have compromised development, lowered resistance to disease and, therefore, allowed easy infiltration of infective agents. The eggs were initially to have been sacrificed prior to hatching. However, after they had hatched it was decided to monitor the apparently disease free hatchlings for later signs of infection, reduced growth or loss of condition.

The failure of eggs experimentally infected by dipping (entry through the egg shell theorised as being natural mode of entry) may be related to the age of the embryo. Kunert *et al* (1993) attempted to experimentally infect snake eggs with *F. solani* isolated from naturally infected eggs. Using cultured mycelia with conidia present, eggs were inoculated three days after oviposition but despite almost 100% ambient humidity in the incubator, the fungus did not penetrate the egg. However, when conidia were transferred to eggs within six hours of oviposition, fungal invasion occurred in all cases, and was macro- and microscopically similar to the original natural process. Kunert *et al* (1993) suggested from their results that *F. solani* could only infect newly laid eggs still moist with oviducal mucus. Further tests by Kunert *et al* (1993) showed that common to many soil fungi, *F. solani* had strong proteolytic and lipolytic activity which was believed to have allowed the penetration of the shell.

In the crocodile study reported here, hyphal and conidial size of *F. solani* would also allow easy penetration through the very much larger shell pores. Kunert *et al* (1993) also suggested that this proteolytic activity may also account for coagulation of the yolk when infected by hyphae.

Kunert *et al* (1993) suggested that the source of fungal infection in snake eggs with the causal agent *F. solani* was most probably the contaminated terrarium in which the adult snake had lived. In the crocodile egg study reported here, fungal contamination occurs immediately after laying when the moist egg is surrounded by a thick, sticky oviducal mucus. Even freshly laid eggs collected immediately after deposition have large amount of organic matter (soil, sand, leaf litter *etc.*) stuck to the mucus. Cooling of the eggs may occur while the female crocodile finishes laying and before she covers the nest and eggs with the nesting material. This natural nesting material is a natural reservoir for fungi, and conidia and hyphal fragments may be drawn into the egg through the cooling process. If eggs are left in the nest for too long, conidia may penetrate the shell through the pores, and organic debris may occlude some pores resulting in the 'speckled' effect seen in some clutches. By light microscopy and scanning electron microscopy studies the dark 'speckles' on the shells were clearly identifiable as small clumps of organic debris including hyphal fragments. When left to grow, fungal sporodochia formed in the pores on the external surface of the shell, with hyphae linking the sporodochia. 'Speckled' eggs are most likely to result in full infection as disinfection with Arocide® only removes contaminants from the exterior of the egg. This disinfection does not remove contaminants which have penetrated to between the shell and the inner membrane. Subsequent incubation provides the ideal growth situation for the fungi as well as for the developing egg.

The 'speckling' of eggs as described in this study has also been seen in photographs of *C. porosus* eggs collected from the wild and incubated under controlled conditions. *C. porosus* eggs with emerging hatchlings shown in the coloured photograph on page 151 of Webb and Manolis (1989) appear to be 'speckled' in a similar way to some eggs collected at Koorana and may, therefore, be contaminated with fungi and bacteria. In contrast, the eggs with emerging hatchlings of *C. johnstoni* excavated from natural nests and shown in coloured photographs on page 104 of Webb and Manolis (1989), did not have the same contamination. In the text edited by Webb,

Manolis and Whitehead (1987) two coloured photographs of *C. porosus* eggs with emerging hatchlings both show eggshells similarly 'speckled' to those found to be infected in this study. A third photo depicting an egg with the shell cut away to view the developing embryo is also 'speckled'.

Kunert *et al* (1993) suggested that the proteolytic and lipolytic activity of *F. solani* allowed it to utilise components of the shells as well as the egg albumen and yolk for its growth. In crocodile eggs the 'speckling' effect and fungal growth was predominantly around the banded section of fertile eggs where the developing embryo and the majority of nutrients are located.

An interaction between the eggs of the Brackish Water Turtle (*Malaclemys terrapin*) and the roots of dunegrass (*Ammophila breviligulata*) was reported by Stegmann *et al* (1988). The grass roots were using buried turtle eggs as a nutrient supply with proliferation of fine laterals around the egg and, rather than penetrating the eggs, the roots usually remained on the outside of the shell. Gamma spectroscopy and the use of isotopes showed that nutrients were moving from the live egg to the live plant. Affected turtles either died during development or failed to hatch because of inability to escape through the confining mass of roots. In this crocodile study the fungus appeared to be using the egg as a source of nutrients and this caused stress on the developing embryo therefore further compromising its growth. If the embryo can survive this loss of nutrient it hatches as a malnourished and undersized animal, similar to the snake hatchlings reported by Kunert *et al* (1993). The young hatchling may be immunocompromised even within the egg and is most likely carrying a latent fungal infection at emergence. Any subsequent stress within the first twelve months of life may be enough to allow the latent fungal infection to become fully established internally and also precipitate manifestation of external symptoms not previously evident.

Genthner and Middaugh (1992) were prompted to study the effects of a normally entomopathogenic fungus, *B. bassiana,* on non-target organisms (fish embryos) after noting *B. bassiana* infections of poikilothermic vertebrates (Fromtling, Kosanke *et al*, 1979; and Georg *et al*, 1962). Genthner and Middaugh (1992) reported that when the developing fish embryos were exposed to conidia of the fungus, embryo rupture and death was observed. However, embryo rupture did not always result in death nor was

death always associated with embryo rupture. It was suggested that the proteinaceous nature of the chorion probably rendered it susceptible to digestion by the fungus causing it to rupture. However, since the effects were observed on living fish eggs the fungal growth was not merely saprophytic. It was concluded that germination and penetration of the chorion by hyphae was sufficient to cause rupture and, therefore, the increased incidence of embryo death. Saprophytic fungi (*eg. Saprolegnia* spp.) commonly colonise dead fish eggs (Neish and Hughes, 1980 and Smith *et al*, 1985, both cited in Genthner and Middaugh, 1992) but *Saprolegnia* spp. hyphae also colonise the chorion of live eggs (Smith *et al*, 1985, in Genthner and Middaugh, 1992). The structure of the crocodile egg consists of an external calcareous shell with an inner shell membrane, and inside the membrane is a chorioallantois which encompasses the albumen (Manolis *et al*, 1987). Inside the albumen, the yolk is surrounded by a thin vitelline membrane to which the developing embryo is attached (Manolis *et al*, 1987). The eggs of *C. porosus* at laying consist of (a) 48.1% yolk, which is composed of water (56.4%), protein and lipids; (b) albumen, which is 96.4% water and the remaining solid portion almost entirely protein; and (c) sub-embryonic fluid, also described as 'thin" albumen (Manolis *et al*, 1987). The highly proteinaceous nature of the crocodile egg, similar to the fish eggs in the report by Genthner and Middaugh (1992), and the snake eggs reported by Kunert *et al* (1993), probably also encourages and sustains hyphal growth without causing death. In this crocodile egg study, *F. solani* was isolated from extra-embryonic membranes after the emergence of live hatchlings as well as from infertile eggs, early embryonic discs and dead embryos. It was not considered to be a saprophytic fungal invasion because of the continued hatchability of infected eggs.

Sub-shell airspaces and sub-membrane airspaces are relatively common in *C. porosus* and *C. johnstoni* eggs (Manolis *et al*, 1987; Whitehead, 1987) although embryos do not appear to breathe air within the sub-membrane airspace (Whitehead, 1987). Crocodilian embryos may remain in the egg for up to two days after pipping, with the delay between pipping and emergence possibly providing a transition from chorioallantoic to pulmonary respiration (Whitehead, 1987) In the study reported here, *F. solani* was regularly isolated from the sub-shell airspace of eggs with almost full term embryos which failed to hatch. It is possible therefore, that full term embryos

which have pipped but not yet emerged may be in an enclosed environment and breathing from a confined airspace which is highly contaminated with fungal hyphae and conidia. Inhalation of the conidia would be almost inevitable, and could explain subsequent fungal infections in the lungs of hatchlings whose deaths may be precipitated by chronic or acute stress during their first year, but whose indoor environment greatly reduces the possibility and probability of inhalation of airborne conidia.

Although mycotoxins are produced by some strains of *Fusarium* species, including *F. solani*, (Nelson *et al*, 1983; Drysdale, 1984; Bosch *et al*, 1989; Roffe *et al*, 1989; Beri *et al*, 1991; Bonner, 1995) this was not probable in the crocodile egg infections since not all embryos were subject to mortality. Similarly, Kunert *et al* (1993) reported that in fungally infected snake eggs, embryos survived in close association with *F. solani* mycelium and, therefore, the presence of mycotoxins was not probable in that situation.

Post-mortem results were not entirely as expected for some of the groups (refer Tables 25 and 26, section 3.13). Groups A (two out of three), E, and G showed positive results to fungal presence. All of these animals had been exposed to viable conidia so this result was not unexpected. Groups B, H, and J showed negative results and had been exposed to non-viable conidia. Therefore no infection had been expected. Group I eggs were not expected to give positive results. However, cooling of this group's eggs may have inadvertently occurred so that the spore suspension was drawn into the egg during the cooling process even though the suspension temperature was initially at the same temperature of the egg. Additionally, although the animal euthanased was reasonably thought to belong to that group, the possibility exists that it did not, for the reasons mentioned previously. Groups D and F were both expected to be negative but results showed positive. This is most likely again due to error in identification of the animals. Other reasons could be that the animals had become infected by other means after the commencement of the trial. Inoculation with contaminated but previously non-viable conidia was not considered possible as test plates had been set up after all inoculations were completed to confirm the state of the conidia. No animals could be positively identified as coming from group C. Because other animals from the two trials could not be identified with absolute certainty, euthanasia and results of further

post-mortems would have been meaningless. The value of the animals was considered to be more than the value of any data so obtained.

Organs from the trial animals which proved positive mycologically, *ie.* the lung, liver and kidneys, were also the organs which were most frequently diseased in those animals infected by natural means. One might perhaps conclude from this data that these are the first organs which become infected by fungal disease. All groups other than D, F, I, and C, according to the data presented, fulfilled Koch's Postulates. However, the sample size was arguably too small for these last two statements to be valid assumptions.

Taking into account the discussions of Hanson (1969) and Wobeser (1994) it was surprising to have elicited any disease response from the animals involved in the Koch's trials. This was because of the considerable positive changes which had been effected on the animals and also to their environment and which, while they would have contributed to any natural disease process prior to commencement of this study, no longer applied to animals in the trial situation. Temperature stress, nutritional stress, density and other social stresses had all been greatly reduced, thus furnishing a situation in which fewer non-trial animals were infected naturally.

From Table 24 it can be seen that hatchling survival as at 31 December of each year has improved from 1990 to 1994. These data have been calculated as a percentage of those animals which originally hatched and not as a percentage of fertile eggs nor a percentage of total eggs laid. Unrelated deaths in 1992 were considered to be the result of a heating failure and subsequent cold stress. Post-mortem results gave no pathological reason for death. (Pierce and Hill, 1992). Unrelated deaths in 1993 were of hatchlings which had been used in a trial. These were then euthanased.

From Figure 9 it can be seen that whichever method of comparison was used (*ie.* as a percentage of total eggs laid, or as a percentage of fertile viable eggs) the hatchability for the 1993/1994 breeding season was approximately double that for each of the preceding five years. The percentage of hatchlings surviving at the end of their first year in 1994 has been approximately doubled. There were a number of contributing factors which may have combined to produce this increase. A reduction in hatchling density in the pens, additional covered retreats and extra feeding stations in each pen possibly may have reduced the amount of territorial or feeding fights. Fewer wounds

were apparent and, therefore, there was a reduced chance of fungal infection through wounds. This also resulted in a higher skin quality. Improved control of the pen temperature with heated air and water, along with a better cleaning regime contributed to reducing external stresses on the hatchlings. A more reliable supply of fresh meat was arranged to reduce sudden dietary changes, and vitamin and mineral supplements were included on a regular basis.

During the 1991/1992 and 1992/1993 breeding seasons (egg-laying November to March, hatching February to late May) investigation of methods to remove fungal spores from freshly laid eggs without damaging the developing embryo were trialed. Attempts at ascertaining the method of entry through the shell were also carried out. Previously the aim had been to determine how the pathogen invaded live hatchlings and yearlings. Another question raised was whether eggs and hatchlings in the wild were affected by fungal disease. No opportunity arose to sample 'wild' nests and eggs in the local region.

Scrapings from the oviducts of captive laying females were not carried out. This cannot be overlooked as a possible source of fungal infection and source of egg contamination. The oviducts from a breeding female, which while defending her nest had been killed by another crocodile, became available for mycological culture. Results were inconclusive due to tissue contamination during the skinning and dissection process.

Systemic mycoses, reported in a wide range of animals from different phyla, are usually highly virulent, difficult to treat and frequently fatal. An oviducal infection sufficiently well established internally to infect developing eggs in a breeding crocodile, could be expected to cause infertility at least, or early death at worst. The probability of all or the majority of female crocodiles on the farm carrying the same and multiple internal mycoses of the reproductive system year after year would be very low.

The results for egg anti-fungal treatments commenced in the 1991/1992 breeding season were initially disappointing. However, it was considered that the method of cleaning the eggs by 'cold washing' had actually contributed to embryo mortality by setting up a temperature gradient, which helped draw fungal inocula from the cooler water into the warmer egg. The extra handling involved in hand washing the eggs of

debris also contributed to embryo mortality due to mechanical stresses set up around the embryonic disc. Also, a heating malfunction due to a power surge after a cyclone was responsible for some egg mortality. The method of using dry vermiculite for the incubation medium contributed to desiccation of some eggs and subsequent mortality, even with high humidity levels in the incubation room.

During the 1992/1993 breeding season, the use of moist vermiculite to incubate the eggs eliminated the possibility of desiccation. This initially created the opposite extreme, with some eggs from the earlier incubated nests swelling to the point of cracking the shell. Transferring the eggs to fresh trays of damp vermiculite which had been partially dried in sunlight prevented further swelling. Humidity levels were maintained at a consistent 98 to 99%.

Hatching success was further improved in the 1993/1994 breeding season. Using warm water to wash the eggs followed by the warm antifungal treatment reduced the possibility of introducing bacterial cells or fungal inocula through the pores of the shells by back-flow due to a temperature gradient. A reduction in egg handling by using irrigation rather than hand washing reduced embryo mortality due to mechanical shear stresses. The overall live hatchling production for that breeding season was 50% to 60% of the total eggs laid, or 61% to 72% of viable fertile eggs (Refer Tables 22 and 23). This compares favourably with the industry hatchability standard of around 70 to 80% of fertile eggs (Lever, pers. comm., Webb *et al*, 1994). Hatchability of *C. porosus* eggs in the wild has been estimated at 25% (Webb and Manolis, 1989). High embryo mortality from nests twenty-four to thirty-two laid late in the 1993/1994 breeding season was partially attributed to eggs physically damaged during and after laying by inexperienced breeding females. Overheating, desiccation and physical damage to eggs laid on bare ground or in the water of the breeding lakes by females which did not build nests also contributed to egg mortality. These eggs were not expected to develop but were processed and incubated with viable eggs. A high level of infertile eggs (14.6% of total laid) was also experienced in this latter part of the breeding season, although overall infertility was less than that recorded in the previous two breeding seasons (19.4% and 20.2%). Eggs designated as infertile were those which after a minimum of one week of incubation had no opaque spot or banding visible on the shell, and when the egg was candled there was no

subembryonic fluid or embryonic disc and the yolk was still floating in the centre (Webb and Manolis, 1987).

Hatchling growth at Koorana has been enhanced. Data prior to this study are not available and yearly comparisons are not possible. The average length and weight for the surviving hatchlings at 31 December 1993 was 745 mm and 1449 g. (Number of hatchlings = 419) The minimum was 360 mm and 66 g which relates to an animal in the sick bay. The maximum was 980 mm and 3542 g. The ages for that group at 31 December 1993 ranged from seven and a half months to eleven months. The largest hatchling was from a clutch which hatched on the 9 March 1993, and was just under ten months old at the census date. The yearly census date of 31 December was chosen as this coincides with yearly stock checks for permit reports to the Queensland Department of Environment and Heritage. These growth rates exceeded those of hatchlings used in trials at another research facility (Mayer, 1994), and on other farms *eg.* Queensland farm, hatchlings at age 12 months, length 500-600 mm (Onions, pers. comm.); Northern Territory farms, hatchlings at age 12 months, 675-696 mm (Webb *et al*, 1994). The average size for twelve month old hatchlings in the wild has been reported as being 730 mm and 870 g (Webb and Manolis, 1989). If the growth rates for Koorana hatchlings can be maintained, culling and skinning could be carried out by the end of the crocodile's second year of life. Culling is normally carried out at age 2.5 to 4.5 years, or 1.5 to 2 m body length, with a skin size belly width of 35 to 40 cm (Lever, pers. comm., McKelvie and Treadwell, 1991). This earlier culling would result in significant cost savings to the farmer as this would eliminate the necessity of maintaining the animals in a heated environment for a third or possibly fourth winter.

4.1 Future Investigations

- **Future research involving immunological studies** could be instigated to determine whether some of the healthy crocodiles or diseased survivors have antibodies against fungi, and whether the development of antigens is a viable option. Mass injections (of perhaps more than one fungal antigen) of farmed crocodiles would be a time consuming and relatively expensive operation and would only be cost effective if there was a proven advantage in the prevention of disease. **No serological or immunological studies were attempted during the period of this research program.** Serological tests are available as aids in the

diagnosis of some mycoses in humans (Buckley, 1971; Collins and Lyne, 1976; Drouhet, 1989; de Repentigney, 1989), including aspergillosis. However, no known serological tests are available for the mycoses reported in this study or for the range of mycoses reported by other workers (refer section 1.4). Frye (1991) reported that few clinical trials had been reported to test the efficacy of induced immunity in reptiles, although autogenous bacterins developed from suspensions of killed bacterial culture had been used for both treatment and prevention of homologous bacterial infections in snakes with promising results (Addison and Jacobson, 1974; Jacobson *et al*, 1985; Kiel, pers. comm.; all cited in Frye, 1991). Frye (1991) also stated that many naturally occurring antibodies to a very wide range of pathogens had been found in reptiles but crocodiles were not specifically mentioned. This may be the case in some of the crocodiles reported here which remained apparently disease free during epidemics.

- **Further biochemical and haematological studies** are necessary to build up a blood profile of both healthy and diseased animals as well as of different age groups.
- **Mycological studies of eggs and nests in the wild** could be useful in determining hitherto undetermined embryo and hatchling mortality in that situation.
- **Mycological studies of the oviducts of breeding females** would ascertain whether mycotic infection is passed on by the female before or during shell deposition. This would be difficult and physically dangerous for the investigator if using live animals, and would cause stress to the female crocodile and possible loss of eggs due to premature laying or egg resorption. Euthanasing valuable breeding female crocodiles would not be a viable option for most crocodile farmers and the only likely source of tissue for investigation would be from animal post-mortems.

4.2 Conclusions

- It is proposed that the fungal disease problem experienced by this crocodile farm arose from early infections of the eggs.
- Fungal conidia of the identified pathogens are ubiquitous in the farm environment and readily adhered to the freshly laid eggs in the nest.

- Scanning electron microscopy studies showed that fungal conidia and hyphae could easily penetrate the egg through the shell pores and calcite layers.
- Optimum egg incubation temperature and humidity favoured fungal proliferation as well as embryo development. Fungi grew within the egg and killed some embryos..
- Once bacteria or fungal conidia have penetrated past the external shell to the membrane layer within the egg, the embryo has a much higher probability of developmental failure.
- Those embryos that hatched and were diseased were smaller than what are now known to be average size(weight and length) and, furthermore they did not grow as large or as rapidly as apparently disease free hatchlings.
- Some animals carry a latent infection and later stresses (environmental, physical, social) precipitate clinical signs of mycoses. Susceptible animals succumb quickly to the infection with death occurring rapidly after the first onset of physical symptoms.
- Treatment of diseased hatchlings is difficult due to the nature of the species.
- To reduce the contact time of the pathogens with the egg, and also to reduce the chance of mechanical damage after the embryo has attached to the inner membrane, it is crucial that nests are robbed soon after the eggs are laid.
- The preventative measure of egg decontamination before incubation had a high success rate in reducing embryonic and hatchling mortality.

4.3 Recommendations and Benefits

As a result of this study the following recommendations are suggested for farmers or other managers of captive breeding crocodilians:-

- early or immediate collection of freshly laid eggs to reduce the exposure time to fungal elements in the nest;
- streamlined cleaning and disinfection of eggs with reduction in manual handling;
- incubation in a separate facility of those eggs already contaminated when they are collected (*ie.* eggs previously described as 'speckled'), as well as cracked or otherwise damaged eggs, to avoid cross-contamination of 'clean' eggs;
- critical control of temperature and humidity with fail-safe alarms and back-up generators in the event of power failure;

- vigilant attention to cleanliness in all areas with regular scrubbing of both animal housing and incubation facilities, followed by fungicidal spraying;
- provision of footbaths at entrances to both hatchling and incubation facilities;
- provision of food supplements for crocodiles of all ages, particularly for hatchlings and yearlings to accelerate growth rates and maintain prime body condition. This is especially significant for breeding females to allow maintenance of body calcium stores and assist in production of nutritionally sound eggs;
- reduction and/or elimination of environmental and/or social stresses on hatchlings;
- investigation of the causes of egg and hatchling mortality are important. If fungal infections are suspected, advise the veterinarian and request that additional appropriate procedures be followed. Veterinary mycology is regarded as a specialist field and mycological testing of tissue is not normally carried out from a standard post-mortem examination and necropsy.

During the course of this research carried out at Koorana Crocodile Farm from 1990 to 1994 inclusive, the following were achieved.

- **Preventative measures reduced infection rates** in both eggs and hatchlings to an acceptable level.
- **Egg hatchability was approximately doubled.**
- **Hatchlings were larger and healthier** than previously observed.
- **Survivorship of hatchlings** to one year of age **was approximately doubled**.
- The **net effect** for the commercial farm in real terms was a **quadrupled output** which had beneficial financial implications for the future.
- **Accelerated growth rates** combined with the increase in the number of yearling crocodiles assured the future commercial aspect of skin production, where previously the enterprise had been highly dependent on income related to tourism.
- **Culling of crocodiles for skin production will be doubled to ten animals per week** in 1996, compared with the 1995 rate of five per week (Lever, 1996). Other plans are in progress for expansion of manufacturing activities, farm-stay holidays for tourists, and consideration being given to opening an abattoir on the farm (Lever, 1996).

5. APPENDICES

Appendix 1

Glochlor bleach

Available chlorine 40 g/l.

Used diluted in water according to cleaning application.

Nitrofurazone Rural Chemical Industries Pty. Ltd., Glenorie.

Antimicrobial powder, usage @ 10 mg/l as immersion bath.

Tecto®/Mertect® Merck Sharp and Dohme, Rockhampton.

Wettable powder, active ingredient thiabendazole, 90% w/w.

Anthelmintic, fungicide. Usage @ 50 mg/kg body weight as fluid drench or on food.

Arocide® Rural West, Perth.

A proprietary mixture of

alkylbenzyldimethyl ammonium chlorides

pentane-1,5-dial (*ie.* glutaraldehyde)

and a concentrated deodorant.

Viricide, fungicide, bactericide and sporocide. Liquid concentrate, usage @ 1:150 as aqueous solution for cleaning application.

Virkon S® Janos Chemicals (NSW) Pty Ltd, Forbes.

A balanced, stabilised blend of peroxygen compounds, organic acids, a surfactant (a salt of a straight chain alkyl benzene sulphonate) and an inorganic buffer.

Viricidal disinfectant. Powder, usage 0.25-1.0% aqueous solution for cleaning application.

Malachite Green Rural Chemical Industries Pty. Ltd., Glenorie.

Antimicrobial powder, usage 1-5 ppm for flushing in open circulation, or 0.5 ppm for immersion bath.

Tincture of iodine

Iodine	20 g
Potassium iodide	30 g
95% ethanol	to 1.0 l

For use as topical antiseptic.

Concentrated glucose solution

Glucose	100 g
Sterile water	100 ml

Refrigerate, dose at 3 ml/kg body weight as fluid drench.

Tricon®

Chlor-tetracycline hydrochloride 55 mg/g.

Used diluted in water as anti-bacterial wash.

Aroclenz® Rural West, Perth.

A proprietary mixture with active ingredients

propylene glycol

benzoic acid

salicyclic acid

propionic acid

malic acid

teric(G'N-8)

gentian violet

Liquid, usage as a wound treatment by topical spray application.

Appendix 2

Food supplements

Calcium di-phosphate.

Calcium pyrophosphate, $Ca_2P_2O_7$, food supplement.

Sprinkled on food at ratio of 50 g/kg body weight.

Feramo D® Vetsearch International.

A proprietary blend of vitamins, minerals, yeast, amino acids and trace elements.

Used as a conditioner for pet, stud and show dogs; during pregnancy and lactation.

Powder added to food at varying rates according to dog size.

Vita-Stress® Pfizer Agricare Pty. Ltd., West Ryde.

A concentrated blend of thirteen vitamins balanced to meet the requirements of livestock under stress. Powder added to food or water at varying rates according to animal species and size.

Pet-Vite® I.G.Y. Manufacturing Pty. Ltd., Hurstville.

A balanced powder formula of twelve vitamins and ten minerals, added to food or water at varying rates according to animal species and size.

Enzactiv® Rural West, Perth.

A proprietary blend of active enzymes in concentrate form which has undergone a process of substrate induction. Includes a wide range of amino acids, vitamins and minerals, and also crude protein, fat, fibre, ash and water.

Dry granular formulation, added to food at varying rates according to animal species and size.

Appendix 3

Neutral buffered formalin (NBF)

Na_2HPO_4	6.5 g
NaH_2PO_4	4.0 g
Formaldehyde	100 ml
Distilled water	900 ml

Bouin's fixative

Saturated aqueous picric acid	75 ml
Formaldehyde	25 ml
Glacial acetic acid	5 ml

Slide adhesive

Gelatine	2 g
Potassium dichromate	2 g
Distilled water	100 ml

Tissue processing schedules - Histokinette

1. Fixation (Neutral Buffered Formalin) 24-48 hours

Washing in distilled water	1 hour
Dehydration in 30% ethanol	1 hour
Dehydration in 50% ethanol	1 hour
Dehydration in 70% ethanol	1 hour
Dehydration in 100% ethanol	1 hour
Clearing in 50:50 ethanol/X3B	1 hour
Clearing in X3B	1 hour
Impregnation with wax I	1 hour
Impregnation with wax II	1 hour
Wax under vacuum	0.5 hour

2. Fixation (Bouin's Fixative)	48-72 hours
Washing in 70% ethanol with Li_2CO_3	1 hour
Washing in 70% ethanol with Li_2CO_3	1 hour
Washing in 70% ethanol with Li_2CO_3	1 hour
Dehydration in 70% ethanol	1 hour
Dehydration in 100% ethanol	1 hour
Clearing in 50:50 ethanol/X3B	1 hour
Clearing in X3B	1 hour
Impregnation with wax I	1 hour
Impregnation with wax II	1 hour
Wax under vacuum	0.5 hour

Appendix 4

Half strength potato dextrose agar (HPDA)

Potato dextrose agar	19.5 g
Bacteriological agar	8.0 g
Chloramphenicol	0.2 g
Distilled water	1.0 l

Potato dextrose agar - Gibco M03940, Oxoid CM139, BBL 11550

Bacteriological agar - Gibco 00001, BBL 12304

Chloramphenicol - Sigma C0378

Sabouraud's Agar Gibco 04200, BBL 11584

Sabouraud's agar	65.0 g
Distilled water	1.0 l

Malt Extract Agar (MEA) Oxoid CM59, BBL 11403

Malt extract agar	50.0 g
Distilled water	1.0 l

Czapek Yeast Extract Agar (CYA)

K_2HPO_4	1.0 g
Czapek concentrate	10 ml
Yeast extract (BBL 11929)	5.0 g
Sucrose	30.0 g
Bacteriological agar	15.0 g
Distilled water	1.0 l

Czapek Concentrate (refrigerate)

$NaNO_3$	30.0 g
KCl	5.0 g
$MgSO_4.7H_2O$	5.0 g
$FeSO_4.7H_2O$	0.1 g
$ZnSO_4.7H_2O$	0.1 g
$CuSO_4.5H_20$	0.05 g
Distilled water	1.0 l

25% Glycerol Nitrate Agar (G25N)

K_2HPO_4	0.75 g
Czapek concentrate	7.5 ml
Yeast extract	3.7 g
Glycerol (AR)	250.0 g
Bacteriological agar	12.0 g
Distilled water	750 ml

Acid Fuchsin in Lactic Acid

Acid fuchsin (C.I. 42685)	0.1 g
Lactic acid	100 ml

This solution kills, fixes and stains.

Appendix 5

Haematological Tests		
Abbreviation	Description	Expressed as
WBC (x 10^9/L)	Leucocyte count	number per litre
RBC (x10^{12}/L)	Erythrocyte count	number per litre
Hgb (g/L)	Haemoglobin	grams per litre
Hct or PCV	Haematocrit or Packed cell volume	volume percentage
MCV (fL)	Mean corpuscular volume	femtolitre = 10^{-15}L
MCH (pg)	Mean corpuscular haemoglobin	picogram = 10^{-12}g
MCHC (g/L)	Mean corpuscular haemoglobin concentration	grams per litre
RDW (%)	Red cell distribution width	percentage
PLT (x 10^9/L)	Platelets	number per litre
PCT (%)	Platelet haematocrit	percentage
MPV (fL)	Mean platelet volume	femtolitre = 10^{-15}L
PDW (%)	Platelet distribution width	percentage

Biochemical Tests		
Abbreviation	Description	Expressed as
A/G ratio	Albumin/globulin ratio	
CPK or CK (IU/L)	Creatine phosphokinase	Int. units per litre
GGT (IU/L)	Gamma-glutamyl transferase	Int. units per litre
AST (IU/L)	Aspartate aminotransferase	Int. units per litre
GLDH (IU/L)	Glutamate dehydrogenase	Int. units per litre
ALT (IU/L)	Alanine aminotransferase	Int. units per litre
LDH (IU/L)	Lactic dehydrogenase	Int. units per litre
CO_2 (mmol/L)	Carbon dioxide	millimoles per litre

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7. PUBLICATIONS AND PRESENTATIONS

Refereed Publication in Journal with Editorial Board and of International Standing

Hibberd, E.M.A, and Harrower, K.M. 1993. Mycoses in Crocodiles. The Mycologist 7(1):32-37.

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Hibberd, E.M.A. and Harrower, K.M. 1991. Mycoses in Crocodiles. Proceedings of the Intensive Tropical Animal Production Seminar, Townsville, Australia, 7-8 August 1991, pp216-223.

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Hibberd, E.M.A. 1992. Fungal Disease in the Saltwater Crocodile, *Crocodylus porosus*. Veterinary Workshop Seminar at the 11th Working Meeting of the IUCN Crocodile Specialist Group, Victoria Falls, Zimbabwe, 2-7 August 1992.

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