Hatchability of Nile crocodile (*Crocodylus niloticus*) eggs: Association with bacteria and fungi in incubation boxes and in eggs that failed to hatch

By

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Declaration

I, Muhammad Muneeb Rauf, declare that the dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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List of Abbreviations

CFU	colony forming units
Spp.	species
TNTC	too numerous to count

Chapter 1
Introduction

Crocodilians are in high demand by the leather industry for their skins. Crocodilians have been hunted mainly for their skins in the wild, which resulted in various species of crocodilians becoming endangered (Santiapillai and de Silva 2001; Lane 2006; Tosun 2013). So, crocodile farming started under captivity.

Crocodilian skin is a highly valued product, costing hundreds of dollar apiece (Tosun 2013). In South Africa the Nile crocodile (*Crocodylus niloticus*) is raised mainly for the production of good quality skins.

In South Africa, Nile crocodile breeding farms are maintained to produce hatchlings which are raised to a specific length to slaughter for their skins. A great variation of hatching percentage (proportion of eggs that hatched) among the clutches has been observed. Poor hatching percentage causes economic loss.

A poor hatching percentage may be due to eggs not having been fertilised or because concpetuses died. (Conceptus refers to the embryo or foetus together with its embryonic or foetal membranes and the fluid they contain.) Fertilisation may fail due to infertile males, infertile females or a failure of mating to occur. Embryo survival is affected by the mother's age and imbalance of nutrients like fat, vitamins, highly unsaturated fat and protein (Cardeilhac et al. 1991). Early embryonic death in alligator eggs was associated with high concentration of lead in yolk (Lance et al. 2006). Hibberd (1996) reported shell defects in *Crocodylus porosus* eggs. According to Hibberd, a low level of calcium in the ration of breeding females may have caused shell defects and ultimately cause a poor hatching percentage. It is not known whether microbial

contamination of eggs and the environment in which they are incubated affects the percentage of crocodile eggs that hatch. This lack of knowledge provided the stimulus for the current study.

Various bacteria and fungi have been isolated from crocodile eggs and it is assumed that they may cause embryonic death. Bacteria were isolated from shell membranes of unhatched crocodile eggs in Zimbabwe (Foggin 1992). Schumacher and Cardeilhac (1990) found small, brown circular lesions under the shell of American alligator (*Alligator mississippiensis*) eggs, on or near the chorioallantois in affected eggs and isolated various species of bacteria from the lesions.

Various fungal species were isolated from eggs of *Crocodylus porosus* and *Alligator mississippiensis* (Huchzermeyer 2003). Various fungi were frequently isolated from the contents of crocodile eggs (Hibberd and Harrower 1993).

On the crocodile farm at which the current study was done, eggs are placed into boxes which are then placed in incubators until they hatch. On the day that the eggs in a box hatch, experienced farm personnel classify each egg as belonging to one of the seven categories listed below. (Each box included in the current study contained eggs from one clutch only, with different boxes containing different clutches). Figure 1 represents a path diagram showing how the seven categories develop.

Classification of eggs after those capable of hatching has done so:

i. "Unbanded". (The expansion of the chorioallantoic membrane and the mobilisation of calcium for the use of the embryo results in an opaque band formation around the equator

of the egg (Huchzermeyer 2003). An egg will be classified as unbanded if it is either unfertilised, or its embryo died a very young age, before any band has formed.)

ii. "Banded" (the egg did develop a band but failed to yield a full-term foetus, indicating that it was embryonated but the embryo or foetus died).

iii. "Rotten" (the egg is rotten, with the putrefaction making it impossible to determine whether the egg had been embryonated or not).

iv. "Dead in shell" or DIS (the egg contains a late-stage foetus that died inside the shell, perhaps because it could not hatch or due to some other cause of late foetal death).

v. "Cull" (the egg yielded a hatchling but the hatchling was culled, usually because it was malformed).

vi. "Mortality" (the egg yielded a hatchling that died after hatching).

vii. "Hatchling survived" (the egg yielded a hatchling that survived and was kept for skin production).

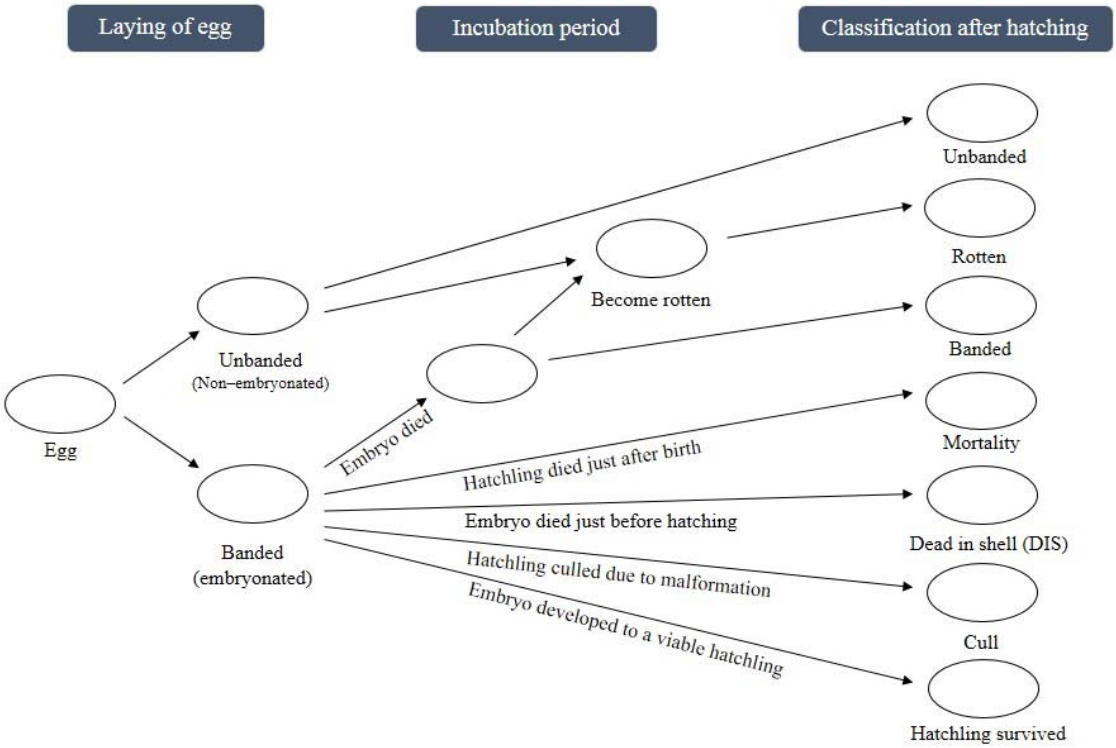

Figure 1: Path model demonstrating the progression of Nile crocodile eggs from ovulation to hatching, and how the different categories of eggs arise

The aim of the incubation and hatching operation on a commercial Nile crocodile farm is to maximise the likelihood of eggs yielding hatchlings that survive and enter the production line (category "Hatchling survived"). Eggs being classified into any of categories i. to vi. will detract from this likelihood. Although there are various causes of eggs falling into categories i. to vi., such as egg shell defects (Hibberd 1996), mother's age, imbalance of nutrients (Cardeilhac et al. 1991), contamination of yolk with lead (Lance et al. 2006) etc, it is not known whether microbial load in incubation boxes before they receive vermiculite and eggs constitutes such a cause. More

specifically, it is not known whether microbial load in incubation boxes affects the development of conceptuses to term.

The aims of this study were to determine

i) Whether eggs are more prone to become lost from the pool potentially capable of yielding conceptuses developing to term in boxes that had more aerobic bacterial colonies and more fungal colonies before they received vermiculite and eggs.

ii) To determine whether there are species of aerobic bacteria, anaerobic bacteria and fungi that are present in unhatched eggs from clutches with low hatching percentage but not in clutches with high hatching percentage, without the converse being true.

It is not known which of the above categories may have been affected by microbial action. We assumed that the microbial load in incubation boxes had no effect on the frequency of eggs remaining unbanded, foetuses developing to term but having to be culled as hatchlings due to anatomical abnormalities (classified as Cull), nor that it caused near term foetuses to die in the shell (DIS), nor that it caused hatchlings to die (Mortality).

We assumed that the microbial load in the incubation boxes may have caused embryos or foetuses to die after band formation has started (the egg being classified as Banded) or it may have caused the egg to have become rotten (classified as Rotten). The response variable "Perhapsloststatus" was therefore created. Perhapsloststatus was an egg-level variable. For each egg that was either classified as Banded or Rotten Perhapsloststatus was assigned the value of one. For each egg that was classified as DIS, Mortality, Cull or as Hatchling survived Perhapsloststatus was assigned the value of zero. Eggs classified as Unbanded were excluded from Perhapsloststatus.

It was assumed that if a species of aerobic bacteria, anaerobic bacteria or fungus is capable of causing the demise of a conceptus it may be found in eggs that failed to hatch from incubation boxes with low hatching percentages but not in eggs failing to hatch in boxes with high hatching percentage. It was further assumed that a microbial species occurring in each of two unhatched eggs from at least one of the boxes with the highest hatching percentages without it occurring in any unhatched egg from any of the boxes with the lowest hatching percentages it is unlikely to cause the demise of conceptuses. This latter assumption will forthwith be referred to as the converse of the first.

Research questions:

1. Is the log odds of eggs being classified as "Banded" or "Rotten" (Perhapsloststatus" = 1) higher than zero ($P < 0.05$) for boxes with more aerobic bacterial colonies or fungal colonies before receiving vermiculite and eggs than boxes having fewer colonies.

2. Is there at least one species of aerobic bacteria, anaerobic bacteria or fungus that occurs in each of at least two unhatched eggs from at least one of the 10 boxes with the lowest hatching percentages that does not occur in any of at least two unhatched eggs from any of the 10 boxes with the highest hatching percentages, without the converse being true?

Chapter 2
Literature review

2.1 Crocodilians

2.1.1 Classification

The members of the family Crocodylidae are referred to as crocodilians. Crocodilians are reptiles (Huchzermeyer 2003). Crocodilians are present around the globe in the tropics and subtropics (Huchzermeyer 2003; Martin 2008). Some of the characteristics of crocodilians, such as their behaviour, heart morphology and fat body distinguish them from other reptiles (Huchzermeyer 2003). Crocodilians include crocodiles, alligators, caimans and gharials.

Lane (2006) stated that 28 species and subspecies of crocodilians are divided into 4 subfamilies: the Alligatorinae (alligator), the Crocodylinae (crocodile), the Gavialinae (gavialis), and the Tomistominae (tomistoma). Crocodilians are the last survivors of the 'Archosaurs' or "ruling reptiles". Today crocodiles look much like they did more than 65 million years ago. Crocodiles are also largest living reptiles in the world (Lane 2006; Nevarez 2009).

2.1.2 The value of crocodilians

Crocodilians play a significant role in the richness of freshwater ecosystems. Crocodilians make deep furrows (Mazzotti et al. 2008) and water holes (Martin 2008) which provide refuge for many crustaceans and arthropods. Crocodiles help to increase fish yield in rivers because they prey on ailing fish more than on healthy fish and their faeces is nutritious for fish (McNeely and Sochaczewski 1988). Blake and Jacobsen (1992) stated that in the past crocodilians were considered to possess little value in the wild except for their skins. Now they are not only highly

admired for the production of quality skins for leather industry and meat but also for their contribution to species richness and population density of fish. Farming with crocodilians also provides tourist attraction. Scientifically they are of great interest because they are the only surviving members of the long extinct archosaurian reptiles. Crocodilians have also been used as animal models for research and teaching purposes, e.g. with respect to neuroanatomy and neurophysiology (O'Rourke and Schumacher 2002). Alligator embryos, for example, can more easily be manipulated than mammalian embryos, making them good models for biomedical studies.

2.1.3 Historical Perspective

Crocodilian populations declined due to the high demand for their skins and meat (Santiapillai and de Silva 2001; Lane 2006; Tosun 2013) and destruction and loss of their natural habitat (Amarasinghe et al. 2015; Blake and Jacobsen 1992; Lane 2006; Martin 2008). When crocodilians were considered as endangered species, hunting was limited or exports banned in different parts of the world (Thuok and Tana 1994; Lane 2006).

Being endemic and vulnerable, Nile crocodiles (*Crocodylus niloticus*) are reared in Africa (Branch 1988). Intensive production operations helped to maintain the wild crocodilian populations (Lane 2006; Nevarez 2009).

2.1.4 Intensive production operations (captive breeding)

Ranching entails harvesting crocodile eggs from the wild and incubating them artificially to produce hatchlings that are then raised and slaughtered (Khosa et al. 2012). Crocodile farming, in contrast, entails keeping breeder animals in captivity, collecting the eggs they produce and

incubating them artificially to produce hatchlings (Khosa et al. 2012). Alligators were the first crocodilians to be reared in captivity; eggs were harvested from the wild and incubated on an "alligator ranch", following which the hatchlings were raised for their hide and meat (Nevarez 2009). By 1960 the American alligator, which was considered a threatened species, was maintained by captive farming in USA, where a population of one million was maintained (Nevarez 2009). Hatching percentage (the proportion of incubated eggs that hatch) and juvenile growth were better in captivity than in the wild (Blake and Loveridge 1975). Alligators at breeding farms started to breed at six years of age as compared to 10 to 12 years for wild alligators (Lance et al. 2006).

Countries other than the United States of America, such as Australia, India, Mexico, Papua New Guinea and South Africa that have wild crocodilian populations also maintain intensive production operations (Nevarez 2009).

Commercial crocodilian farms throughout the world are providing economic benefits (Lane 2006; Martin 2008) and are increasing the wild population by releasing the juveniles in the wild (Blake and Loveridge 1975). In contrast to the more developed pig and poultry industries, crocodile farming is still in the growing phase with huge potential for commercialisation (Peucker et al. 2005).

2.2 The global crocodilian industry

2.2.1 Crocodilian skins

The crocodilians are being used mainly for their skins, while meat and teeth are the main by products (Brazaitis 1987; Caldwell 2004). Crocodilian processed skin is highly valued,

sometimes costing hundreds of dollar apiece (Tosun 2013). About 1.36 million crocodilian skins were traded globally in 2011 (Caldwell 2013) almost similar to the figure in 2010 (Caldwell 2010).

2.2.2 Crocodilian products other than skin

Initially meat was considered as a mere by-product of crocodilian farming, but it is now valuable. The annual global production of crocodilian meat has been approximately 400 tonnes since 1990 (Caldwell 2010). Mainly Nile crocodiles, American alligators and Siamese crocodiles are used for meat. Different parts of the crocodile body, like blood, bones and teeth, head and skulls are used by pharmaceuticals, traditional medicines and tourists, respectively.

2.3 The South African crocodile Industry

In South Africa the crocodile industry is limited to *Crocodylus niloticus* (Khosa et al. 2012).

2.3.1 The taxonomy of the Nile crocodile

The Nile crocodile (*Crocodylus niloticus)* occurs in Africa (Huchzermeyer 2003). The scientific classification of Nile crocodile is as follows:

Kingdom:	Animalia
Phylum:	Chordata
Class:	Reptilia
Superorder:	Crocodylomorpha
Order:	Crocodilia
Family:	Crocodylidae

Subfamily: Crocodylina

Genus: *Crocodylus*

Species: *C. niloticus*

2.3.2 A brief overview of crocodile farming in South Africa

In South Africa crocodile farming started in late 1960s (Cott and Pooley 1971; Regional reports… c2002). South Africa has the potential to produce Nile crocodile skins, which, together with skins of four other crocodilian species are in demand by the luxury market. According to Pfitzer (2014) crocodile farming in South Africa is mainly associated with rearing of crocodiles eventually slaughtered for belly skins exported for production of exclusive leather products. Almost 156000 Nile crocodile skins per year were traded globally between 2002–2011 with a major share by South Africa, Zambia and Zimbabwe (Caldwell 2013). Crocodile meat produced in South Africa is either exported or sold to restaurants or used as unprocessed crocodile feed on the farm (Hoffman et al. 2000).

2.3.3 The crocodile egg

The crocodile egg is oblong ovoid in shape and has a hard shell (Huchzermeyer 2003). The egg shell provides calcium for the oviparous embryo (Packard et al. 1992; Stewart et al. 2009; Stewart and Thompson 1993). On average, Nile crocodile females lay about 40 eggs per clutch in captive breeding (Khosa et al. 2012).

2.4 Hatching of crocodile eggs in captivity

2.4.1 Laying season of Nile crocodiles

The laying season varies in different parts of the distribution range of *Crocodylus niloticus:* In Northern Botswana eggs were laid from September until October (Dzoma et al. 2008). In the south eastern part of Zimbabwe Nile crocodile females nested from early September until early October (Kofron 1989). In Northern Kenya, however, laying was reported to occur from the middle of November 1965 until the end of January 1966 (Modha 1967).

Nile crocodile females at a captive breeding farm in the North West province of South Africa lay eggs from the middle of September until the end of November in holes they make in the soil or sand just before laying the eggs. Workers at the farm collect these eggs from a nest by keeping the female away and putting the eggs in polystyrene boxes for incubation. (Immediately before loading a box with eggs a 5-cm thick layer of vermiculite is placed in the bottom of the box). Usually, all the eggs of the same clutch are put into the same box, although, for large clutches, the eggs are placed into two boxes.

2.4.2 Variation in the hatchability of crocodile eggs

It has been observed that large variation exist in hatchability of the crocodile eggs among clutches within a captive breeding farm. Although the fertility rate (the percentage of eggs that banded) of fresh-water crocodile (*Crocodylus johnstoni*) eggs was close to each other at 54.3% and 58.8% on two commercial farms a larger difference (59.1% compared to 82.6%) existed in the precentages of banded eggs that hatched (Peucker et al. 2005). Dzoma et al. (2008) observed variation of hatchability in Nile crocodile eggs ranging from 30% to 90%. It is therefore

indicated to investigate the loss of viable embryonated eggs during incubation. Specifically, it may be of interest to investigate the association between the microbial load of incubation boxes with the proportion of eggs that are either banded or rotten at the time that the remainder of the clutch of eggs have hatched.

2.5 Causes of poor hatchability in crocodilian eggs

2.5.1 Egg shell defects

Huchzermeyer (2003) stated that shell defects may occur due to low calcium levels in the ration of breeding females. Huchzermeyer also stated that excessive moisture in the nest causes eggs to excessively absorb moisture, swell and burst, while the embryos suffocate and die.

Hibberd (1996) reported the following shell defects in *Crocodylus porosus* eggs: soft shells, partial shell formation, absence of the shell with only membranes, additional calcareous protrusions on the external surface of the shell, under- or oversized eggs and deformed eggs with incomplete sealing. According to Hibberd a low level of calcium in the ration of females is believed to be the cause of shell defects ultimately causing low percentages of eggs to hatch.

2.5.2 Microbial contamination of eggs by the environment

Microbial contamination of eggs in different species of birds have been reported to cause poor hatchability and early chick mortality. Higenyi and Kabasa (2014) reported different microbial contaminants like *Escherichia coli, Proteus* spp., *Pseudomonas aeruginosa, Staphylococcus aureus* and fungi in hatching eggs of poultry which caused poor hatching percentages.

Al-Bahry et al. (2009) isolated various bacteria from the eggs of turtles. The most frequent isolates were *Pseudomonas* spp. (30.3%), *Salmonella* spp. (19.2%), *Enterobacter* spp. (14.3%) and *Citrobacter* spp. (13.1%).

Little research has been done on the role of microbial contamination or infection on hatching percentage of crocodile eggs. Salpingitis, oophoritis and endometritis may result from ascending infections originating from intestine or cloaca (Huchzermeyer 2003). When crocodile eggs are placed inside polystyrene boxes which are then placed in the incubators for incubation microbial contamination from the incubation boxes is possible. This possibility provided the stimulus for the current study.

A brief summary of research done on bacterial and fungal infections in general in crocodiles and of the eggs specifically are given in the following section.

2.5.3 Bacterial and fungal species

Following sections present the bacterial and fungal species pertaining to generalised infection of crocodilians and specifically to eggs.

2.5.3.1 Bacterial infections

In crocodiles, septicaemia can easily be diagnosed on post-mortem examination and is commonly associated with a wide range of bacteria (Navarez 2009). Lovely and Leslie (2008) studied the intestinal flora of 29 wild Nile crocodiles and identified 21 isolates as being *Microbacterium* spp., 14 as being *Enterococcus faecalis*, 10 as being *Aeromonas hydrophila* and 9 as being *Escherichia coli*. *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* were isolated from frozen Nile crocodile meat samples (Makanyanga et al. 2014). Chlamydial

infection causes hepatitis in farmed hatchling Nile crocodiles as well as mucous membrane- and systemic infections (Huchzermeyer et al. 1994).

Salmonella arizona, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were isolated from shell membranes of unhatched crocodile eggs in Zimbabwe (Foggin 1992).

Schumacher and Cardeilhac (1990) found small, brown circular lesions under the shell, on or near the chorioallantois in affected eggs and isolated *Enterobacter cloacae*, *Citrobacter* spp., *Proteus* spp. and *Pseudomonas aeruginosa* from them.

Salmonella braenderup, S. anatum, and *Arizona* spp. have been isolated from cloacal swabs and *Corynebacterium* spp. from a tail abscess (Shotts et al. 1972). It is plausible that eggs may become contaminated from such sources.

2.5.3.2 Fungal infections

Twenty different species of fungi have been obtained from 29 wild-caught African dwarf crocodiles, slaughtered for the market (Pare et al. 2006). Hibberd and Harrower (1993) isolated and identified *Fusarium solani* from the tissue samples collected from the lesions of liver, lungs, small intestine and bowel of infected *Crocodylus porosus*.

Different fungal species isolated from eggs of *Crocodylus porosus* and *Alligator mississippiensis* were *Fusarium solani*, *F. oxysporum*, *Paecilomyces lilacinus*, *P. aviotti*, *Penicillium fellucanum* and *Aspergillus* spp. (Huchzermeyer 2003). *Fusarium solani*, *Aspergillus spp*. and *Paecilomyces* spp. were frequently isolated from egg contents of incubating eggs of *Crocodylus porosus* (Hibberd and Harrower 1993). Huchzermeyer (2003) cited Hibberd (1994), who found that the

hyphae and spores can pass through the pores of *C. porosus* eggs, and found growth of hyphae along minute cracks.

2.5.4 Other causes

Cardeilhac et al. (1991) has described various causes of poor hatchability in crocodilian eggs. Embryo survival is affected by the mother's age and imbalance of nutrients like fat, vitamins, antibiotics, highly unsaturated fat and protein. Millstein et al. (1994) observed that the concentrations of various fatty acids differed in the egg yolk of clutches with high- and low hatching percentages.

Early embryonic death in alligator eggs was significantly associated with high concentration of lead in yolk (Lance et al. 2006).

Chapter3
Materials and Methods

3.1 Study site

The study site for this project was Le Croc captive breeding farm located at about 30 km north of Brits in the North West province of South Africa. Nile crocodile breeding stock are kept at the farm. The females lay eggs during September, October and November in the sandy soil next to the breeder ponds. The eggs are collected from the soil and put into the polystyrene incubation boxes immediately after a 5-cm layer of vermiculite was placed at the bottom thereof. One clutch is normally placed in one box but if the clutch is too large the eggs are placed into two boxes. All eggs are incubated artificially in the same incubator room of approximately 15 x 5 x 3 m, with a cement floor and smooth walls and ceiling. The incubator was cleaned and disinfected before the first incubation boxes with eggs were placed therein. Boxes were stacked on racks. The temperature and moisture of the room were regulated at 32.5 °C and 80% relative humidity. Temperature and humidity are maintained by automatic control system. The incubation period of Nile crocodile eggs is normally about 80 days. After the incubation period, hatchlings are collected from the incubation boxes and transferred to environmentally-controlled rearing pens. The crocodiles are then reared in climate-controlled houses with indoor ponds until they reach a length of at least 1.3 m before they are slaughtered for their skins.

3.2 Study animals

No live animal was used in this study. On the day of hatching the outcome classification (Unbanded, Banded, Rotten, Dead in shell, Cull, Mortality or Hatchling survived) of each egg in each incubation box used in the study was determined. Contact agar plate impressions for

microbial load (aerobic bacteria and fungi, as described in Section 3.4.2) were taken from the boxes in which the eggs are incubated and swabs were taken from eggs that failed to hatch for the isolation and identification of aerobic bacteria, anaerobic bacteria and fungi.

3.3 Experimental design

3.3.1 Pilot study

A pilot study was conducted about 3 weeks prior to the onset of the main study to determine the best site inside an incubation box from which to obtain agar plate impression samples during the main study. The aim was to determine the site inside the egg incubation boxes having the highest aerobic bacterial and fungal load. Ten incubation boxes were selected randomly from the store room where the boxes had been stored away since the end of the previous year's hatching season. Each box was then prepared according to the routine before the breeding season, up to the point where they are ready to receive vermiculite and eggs (see Section 3.4.1). Plate agar impressions were taken from 4 sites in each box and the numbers of bacterial and fungal colonies determined at each site, following which the site with the highest mean numbers of aerobic and fungal colonies were determined. This site was subsequently used in the main study.

3.3.2 Main study

The first aim of the study was to determine whether the log odds of eggs being classified as Banded or Rotten (Perhapsloststatus) as zero or one is related to the aerobic bacterial and fungal colony counts in incubation boxes before the eggs and vermiculite were placed in them (Study Main 1). The second aim was to determine whether there are species of aerobic bacteria, anaerobic bacteria and fungi isolated from unhatched eggs from boxes with the lowest hatching

percentages but not in unhatched eggs from boxes with the highest hatching percentages (Study Main 2).

3.3.2.1 Design of Study Main 1

One incubation box containing all or half of the eggs from each of 100 consecutive clutches yielded 100 test boxes that were used in the study. On average, about 24 eggs are placed in a box, rarely more than 40, with a maximum of 45.

From each of the 100 test boxes the aerobic bacterial and fungal colony counts were determined immediately before the box received vermiculite and eggs, following which the box was placed in the incubator. Aerobic bacterial and fungal colony counts were again determined from each box once the eggs therein had hatched.

Then we determined the association between aerobic bacterial colony counts or fungal colony counts (independent variables) and Perhapsloststatus (response variable), using logistic regression.

The pilot study had shown that the number of aerobic bacterial and fungal colonies per agar plate taken from the centre of the floor of boxes vary among boxes (Table 2 and Table 4). It was therefore expected that the numbers of aerobic bacterial- and fungal colonies would vary among the 100 boxes destined for use in Study Main 1. Accordingly, Study Main 1 was a single cohort, cumulative incidence study in which all subjects were followed for the full duration of incubation (Dohoo et al. 2010).

3.3.2.2 Design of Study Main 2

The source population for Study Main 2 was the 100 boxes used in Study Main 1. Species of aerobic bacteria, anaerobic bacteria and fungi isolated from unhatched eggs of 10 lowest hatching percentage test boxes were compared directly with the species isolated from 10 highest hatching percentage test boxes.

3.4 Experimental procedures

3.4.1 Preparation of incubation boxes

Polystyrene boxes (600 mm long by 400 mm wide by 130 mm high) are used for the incubation of crocodile eggs. The same polystyrene incubation boxes are used repeatedly during consecutive incubation seasons on the farm. After each hatching season these boxes are washed, disinfected and stored for the next incubation season.

Starting a few days before the anticipated onset of the laying season, about 10 boxes were prepared each day as follows: Boxes were washed with a stabilised blend of Peroxygen compounds, surfactant, organic acids, and inorganic buffer (Virkon, Neogen Corporation 944 Nandino Blvd, Lexington, ky 40511), and then air dried in full sun light. Once dry, the boxes were sprayed with a 0.35% solution of quaternary ammonium and biguanidine compounds (F10, Health and Hygiene (Pty) Ltd. P.O.Box 347, Sunninghill, 2157 South Africa). The boxes were then air dried and stowed away in the incubation room, each box covered by its lid. This washing and disinfection continued throughout the laying period. A box may have been stowed away for 7 to 10 days before being used to receive vermiculite and eggs. This is because the number of

females laid per day varied. On some days no nest was found in the field whereas on others as many as 15 nests were found.

3.4.2 Collecting and processing specimens for the pilot study

About 4 weeks before the anticipated onset of the main incubation season 10 randomly selected boxes were taken from where they had been stored since the end of the previous year's hatching period. They were then washed and disinfected as described in Section 3.4.1, which is the same as the routine normally used for all incubation boxes prior to being used during the laying and incubation season.

One week after the above preparation the following samples were taken from each of the 10 boxes: Contact agar plates of "Plate count agar" and "Potato dextrose agar" were used for aerobic bacterial and fungal colony culturing, respectively, and the swabs used were sterile, cotton wool swabs for culture. Plates had a diameter of 5.6 cm and contained 15 ml of agar each. Agar was prepared in the Bacteriology Laboratory of the Faculty of Veterinary Science of the University of Pretoria, and the agar plates purchased from Labotech (Halfway House, Gauteng, South Africa). One plate-agar impression for aerobic culture was collected from the under surface of the lid (Site 1), one from the centre of the upper surface of the floor (Site 2), one from next to the corner of two sides and the floor (Site 3) and one from the centre of an opposite wall of the box (Site 4). Another set of agar plates was collected just beside these sites in the same boxes for fungal culture. Agar surface was exposed for a moment while touching it against the site for impression and then again covered with the lid. The samples were kept in polystyrene boxes with icepacks inside during collection and delivery to laboratory.

Each contact agar plate was marked with the box number, site of collection and whether it is for bacterial or fungal culture. Samples were delivered to the bacteriology laboratory of the Faculty of Veterinary Science within 24 h. The bacteria agar plates were incubated at 35 °C and fungal plates at 25 °C.

The numbers of bacterial colonies cultured on each plate were counted after 48 h and fungal colonies after 72 h. Fungal plates were incubated further for one month for slow growing fungi following which another colony count was done. The bacteriology laboratory performed all bacterial and fungal colony counts and the results were used for statistical analysis.

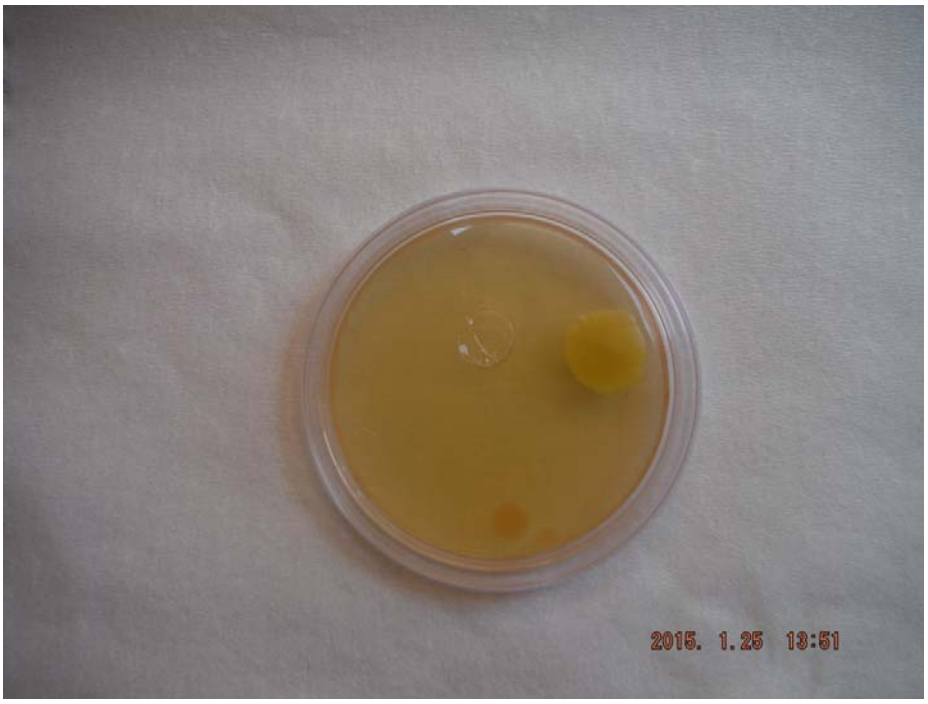

Figure 2: Colony count agar plate with 3 aerobic bacterial colonies

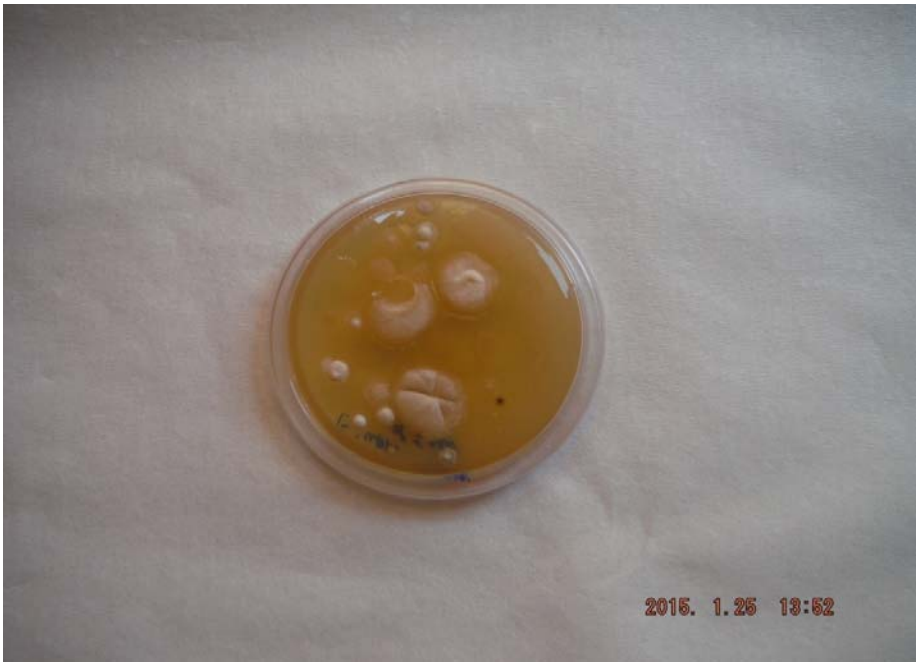

Figure 3: Colony count agar plate with 18 fungal colonies

3.4.3 Collecting and processing specimens for Study Main 1

This study involved 100 clutches (test clutches) laid between 3 and 17 October and between 30 October and 7 November 2014. Each clutch laid during these periods was included in the study. The break from 18 to 29 October was due to the Christmas recess when the laboratory would have been unable to supply fresh agar plates for the assessment of microbial load once the boxes have hatched.

The primary investigator (researcher) learned how to investigate the colony forming units from the agar plates under the supervision of bacteriology laboratory staff so that he could do the colony counts during the Christmas holidays when laboratory personnel might not be available.

3.4.3.1 Before incubation

For the main study one plate agar impression culture for aerobic bacteria and one for fungi was collected next to each other from the centre of the floor of each incubation box. Immediately after collecting the plate agar impressions the floor of the incubation box was covered with a 5-cm thick layer of vermiculite and the eggs of a clutch placed thereon. If a clutch was small enough all its eggs were placed in the same polystyrene incubation box. If the clutch was too large to fit in one box its eggs were divided among two boxes labelled A and B. All the single boxes from small test clutches and the A boxes of large test clutches were used in this study and are henceforth referred to as "test boxes".

After putting the eggs on the layer of vermiculite, they were further covered with vermiculite and the box placed in the incubator for incubation. This was done for each of the 100 test boxes.

3.4.3.2 After incubation

After incubation, once the hatchlings were removed from the box, the unhatched eggs were removed from the box. These unhatched eggs were packed individually in zip lock bags marked with the box number and egg number and further used for Study Main 2 (see section 3.4.4). After removing the eggs from the box the vermiculite was removed by turning the box up side down without touching the inside of the box. Just after removing the vermiculite from the box contact agar plate impressions for aerobic bacteria and fungi were taken next to each other from the centre of the floor of the box. These agar plates were marked with clutch number, box number and transferred to laboratory for incubation. The above–mentioned procedure was done for all 100 test boxes. Culture and colony counts was done as described for the pilot study.

3.4.3.3 Determining which person's counts to use for statistical analysis

For agar impressions made before the eggs were incubated only the primary researcher counted the aerobic bacterial colonies on the plates from some test boxes 48 h after the onset of incubation, only the laboratory counted the colonies on the plates of some other test boxes, whereas both counted the colonies on yet some other plates. The same applies to the number of fungal colonies by 72 h after the onset of culture.

For all test boxes where both the counters counted microbial colonies but neither the primary investigator nor the laboratory found colonies that were too numerous to count (TNTC), we used the count of the primary investigator. If both the counters counted colonies and one found TNTC while the other found a countable number of colony counts we used the latter count. If both the counters classified a test box as TNTC we used the count as TNTC. For all such boxes where only the primary investigator did the count we used his count as the final count, and where the only laboratory did the count we used its count as final count. The statistical analysis to compare the counts obtained by the primary investigator and the laboratory for those plates where both counted the colonies is described in Section 3.5.2.1.

Table 16 under Results shows who did which counts, and whose count was used for statistical analysis. The primary investigator performed all bacterial counts done after hatching as well as all fungal counts one month after the onset of culture and his counts are shown under Results Table 18.

3.4.4 Study Main 2 (culture and microbial species identification in eggs that failed to hatch)

3.4.4.1 Collection of swabs

Swab samples were collected from unhatched eggs from the source population (100 test boxes) to isolate and identify aerobic bacteria, anaerobic bacteria and fungi. For a clutch to be considered at all for culturing of the swabs from unhatched eggs, one must have collected swabs from at least two eggs that failed to hatch. When selecting the boxes with the highest hatching percentages, we could therefore not consider boxes with a 100% hatching percentage or that had only one egg failing to hatch. We selected the 10 clutches with the highest hatching percentages from amongst those that had at least 2 unhatched eggs.

The method was as follows:

An area of approximately 5 cm by 5 cm on the shell of each unhatched egg was encircled with a pencil. The encircled area was sterilised by rubbing it clean with a swab moistened with 99.5% ethanol and keeping the area wet with 99.5% ethanol for one minute, following which the egg was allowed to dry (Adesiyun et al. 2005, Adesiyun et al. 2014, Al–Bahry et al. 2009). The shell was then pierced with a sharp, sterile punch, following which a swab was inserted into the area between the shell and the shell membrane, and through the shell membrane into the albumin and yolk or, if the egg was embryonated, into the core of the egg. Then the swab was sealed and the package identified with the egg number, clutch number and box number (if the clutch was placed in more than one box), and stored at -85 °C until all swabs had been collected. Once the last sample from all the 100 test boxes was collected, the 10 test boxes that yielded the lowest

hatching percentages and the 10 that yielded the highest hatching percentages were selected. The swabs collected from the unhatched eggs belonging to these twenty boxes were cultured to isolate and identify aerobic bacterial, anaerobic bacterial and fungal species.

Figure 4: Piercing an egg shell with a sharp sterile punch

Figure 5: Collection of swab sample from an unhatched egg

The bacteriology laboratory of the Faculty of Veterinary Science processed the selected swabs for isolation and identification of the species of aerobic, anaerobic and fungal microbes.

3.4.4.2 Methodology adopted for species isolation and identification

For the isolation of aerobic bacteria:

1. Used non-selective media i.e. Columbia blood agar + 5% horse blood.
2. Applied a quadrant streaking method.
3. Incubated for 24 h at 37 °C, if there is growth, the colonies were picked up and inoculated as subculture on non-selective media (Columbia blood agar + 5% horse blood) and selective media (MacConkey agar).
4. Incubated for 24 h at 37 °C to get a pure culture.

Pure cultures of bacteria were identified as follows:

1. Lactose or non-lactose fermenter (MacConkey agar).
2. α, β, and ɣ haemolysis (Columbia blood agar + 5% horse blood).
3. Gram staining only from blood agar.
4. Oxidase test (Columbia blood agar + 5% horse blood).
5. Indole test (Columbia blood agar + 5% horse blood).
6. Catalase test (Columbia blood agar + 5% horse blood).

Tests 1–6 are rapid identification tests which allow one to place a bacterium in a group. The bacterium is then further identified to species level using standard biochemical testing (sugar fermentation, hydrolisation and utilisation tests).

For the isolation of anaerobic bacteria:

1. Used Columbia blood agar and incubated at 37 °C in a Bactron anaerobic chamber for 72 h.
2. The plates were checked every 24 h for growth.
3. If some growth was found then sub culturing was done to attain a pure culture.

Pure cultures of anaerobic bacteria were processed as follows:

1. Gram staining.
2. Catalase test.
3. Rapid identification with commercial API system (rapid ID32A bioMeriex® b9280 Marcy I´Etoil France).

For the isolation of fungi:

1. Used potato dextrose agar.
2. Incubated at 25 °C.
3. First look at 5 days.
4. Sub-cultures were made by inoculating new agar plates. A portion of the fungal colony was removed with an inoculation needle and transferred by touching the new agar plate on four spots or areas of the plate.
5. The new plate was then incubated to achieve a pure culture.
6. Fungal staining (Lacto Phenyl Blue staining) for microscopic morphology.
7. The microscopic morphology of the colony was matched with pictures.

3.5 Data analysis

3.5.1 Pilot study

The Kruskal Wallis equality of populations rank test was used to determine whether the four sites in incubation boxes harbours different numbers of aerobic bacteria and fungi. Following that, the six pairwise comparisons among pairs of sites were done using a Wilcoxon signed rank test, and applying the Bonferroni correction to the P-values by multiplying each by 6. The numbers of colonies at two sites were considered significantly different if the Bonferroni-corrected P-value was below 0.05.

3.5.2 Study Main 1

3.5.2.1 Comparison of colony counts obtained by the primary investigator and the laboratory

A two-tailed paired *t*-test was used to compare the aerobic bacterial colony counts obtained by the primary investigator and the laboratory in those 14 boxes where both counted the colonies and neither found that the colonies were too numerous to count.

Similarly, a two-tailed paired *t*-test was used to compare the fungal colony counts after 72 h of incubation at 25 °C that were obtained by the primary investigator and the laboratory in those 31 boxes where both counted the colonies and neither found that the colonies were too numerous to count.

3.5.2.2 Determining the effect of microbial colony counts on the possible loss of conceptuses

The response variable was Perhapsloststatus, as defined in Chapter 1.

The independent variable of interest was Count category, which was compiled as described below.

Aerobic bacterial colony counts after 48 h incubation at 35 °C, as well as fungal colony counts after 72 h incubation at 25 °C, respectively, ranged from 0 to TNTC (too numerous to count). Colony count (a count variable) was grouped into 5 categories (0 to 4) as follows to create the categorical variable "Count category":

0: Colony counts ranging from zero to below the 25th percentile

1: Colony counts ranging from the 25th percentile to below the 50th percentile

2: Colony counts ranging from the 50th percentile to below the 75th percentile

3: Colony counts from the 75th percentile to the maximum that was countable

4: Too numerous to count

Counter (Primary investigator coded 0 and Laboratory coded 1) was included in the model as a second independent variable. It was considered a fixed, potentially confounding effect.

The variable Taxon, coded 1 for aerobic bacteria and 2 for fungi, was used to restrict each model to either aerobic bacteria or fungi.

Clutch was used as the cluster variable (random effect).

The Huber/White sandwich estimators of standard errors were used. This was achieved by means of the "*vce(robust)*" option in Stata.

We used a population-averaged mixed-effect logistic regression model to determine the effect of Count category (first for aerobic bacterial colonies and then for fungal colonies) on Perhapsloststatus. Count category was used as an indicator variable having five categories, with categories 1 to 4 respectively being compared to Category 0, which was used as baseline category. As an example the Stata command for the model to estimate the effect of

Count category for aerobic bacterial colonies on Perhapsloststatus by this method were as follows:

Clutch was first set as the group variable using "*xtset clutch*", followed by

"xtlogit perhapsloststatus i.countcategory counter if taxon==1, pa vce(robust)"

The analyses were first done with the coefficients on the logit (log odds) scale, and then as odd ratios, using the "*or*" option in stata as shown in the next example of a command:

"xtlogit perhapsloststatus i.countcategory counter if taxon==1, pa vce(robust) or"

Fisher's exact test was used to compare the proportion of boxes that had too numerous to count aerobic bacterial colonies before they received eggs with the proportion that had too numerous to count aerobic bacterial colonies after the eggs have hatched. The same was done with respect to the proportion of boxes with too numerous to count fungal colonies.

For all analyses, α was set at 0.05.

Stata 14, StataCorp College Station, Texas, USA, was used for all statistical analyses.

3.5.2.3 Study Main 2

Species isolated from unhatched eggs from the test boxes that had the lowest hatching percentages were directly compared with the species isolated from unhatched eggs from the test boxes that had the highest hatching percentages.

Chapter 4
Results

4.1 Pilot study

4.1.1 Comparison of the number of aerobic bacterial colonies among sites

Table 1 shows the numbers of aerobic bacterial colonies on contact agar plate impressions in each of the four sites sampled in each incubation box used in the pilot study. Most of the colony counts from plate agar impressions taken from the centre of the floor of the boxes appear higher than those of the other sites. As shown in Table 2 there is insufficient support for the null hypothesis of no difference in the number of aerobic bacterial colonies among the four sites in the incubation boxes (Kruskal Wallis test, $P < 0.05$).

The number of aerobic bacterial colonies at the centre of the floor was higher than the number on the underside of the lid ($P < 0.05$) and tended to be higher than on the side wall, near the bottom corner between two neighbouring walls and the floor ($P = 0.07$). The other pairwise comparisons were not significant (Wilcoxon signed rank test, with Bonferroni correction, $P > 0.05$).

Table 1: Number of aerobic bacterial colony forming units at different sites in incubation boxes that were ready to load with vermiculite and eggs

	Site inside the polystyrene incubation box where agar plate impression was done			
Box	Centre of the lid	Centre of the floor	Bottom corner	Centre wall
1	0	2	0	0
2	0	TNTC	1	0
3	0	2	0	0
4	1	3	0	0
5	0	11	0	1
6	0	0	0	0
7	0	1	0	0
8	0	2	0	1
9	0	0	0	2
10	0	1	1	4

Table 2: Rank sums for the numbers of bacterial colony forming units at four sites in each of 10 incubation boxes

Sites	Rank Sum
Centre of the under surface of the lid	146.00
Centre of the floor	303.50
Next to the corner of two sides and the floor	162.00
From the centre of an opposite wall	208.50

Chi–squared = 11.008 with 3 d.f.
Probability = 0.0117
Chi–squared with ties = 14.679 with 3 d.f.
Probability = 0.0021

4.1.2 Comparison of the number of fungal colonies among sites

Table 3 shows the number of fungal colonies on contact agar plate impressions in each of the four sites sampled in each incubation box used in the pilot study.

The Kruskal-Wallis test (Table 4) shows insufficient support for the null hypothesis of no difference in the number of fungal colonies among the sites ($P < 0.05$). As with the numbers of aerobic bacterial colonies, the number of fungal colonies at the centre of the floor had the highest rank. The pairwise comparisons among sites showed that the underside of the lid had fewer

fungal colonies than the centre of the floor ($P < 0.05$), whereas no other pairwise comparison was significant ($P > 0.05$).

The pilot study showed that the microbial load differed among the four sites in incubation boxes and that the centre of the floor (Site 2) of incubation box contained higher microbial loads than some of the other sites. Accordingly, the contact agar plate impressions in Study Main 1 were collected from the centre of the floor (Site 2) of each test box.

Table 3: Number of fungal colony forming units at different sites of polystyrene incubation boxes

	Site inside the polystyrene incubation box where agar plate impression was done			
Box	Centre, lower lid	Centre, inner floor	Bottom inside corner	Centre inner wall
1	0	2	0	0
2	0	1	2	1
3	0	0	1	0
4	0	3	0	0
5	0	0	0	0
6	0	1	0	0
7	0	1	0	1
8	0	1	0	0
9	0	8	0	0
10	1	2	17	9

Table 4: Rank sums for the numbers of fungal colony forming units at different sites

Site	Rank Sum
Centre of the under surface of the lid	146.50
Centre of the floor	289.00
Next to the corner of two sides and the floor	195.50
From the centre of an opposite side	189.00

Chi–squared = 7.920 with 3 d.f.
Probability = 0.0477
Chi–squared with ties = 10.591 with 3 d.f.
Probability = 0.0142

4.2 Study Main 1

Table 16 shows the colony counts of aerobic bacteria and fungi before the boxes were loaded with vermiculite and eggs with the counts of both counters and also shows the total number of eggs, number of eggs classified as Banded or Rotten (Perhapslost = 1) and the number of unbanded eggs in test boxes. (Table 16 is extensive and in landscape format. To facilitate reading, it is placed at the end of Section 4.2 on page 45.

4.2.1 Comparing the colony counts obtained by the primary investigator and the laboratory

The primary investigator counted the numbers of aerobic bacterial colonies and fungal colonies in 78 of the 100 boxes before they received vermiculite or eggs and the laboratory personnel performed these counts in 55 and 62 of the boxes.

The primary investigator and the laboratory attempted to both count the aerobic colonies on agar plates taken from 33 boxes before they received vermiculite or eggs (Table 16). Nineteen of these boxes contained colonies that were too numerous to count. The primary investigator and the laboratory also attempted to both count the fungal colonies on agar plates taken from 40 boxes before they received vermiculite or eggs (Table 16). Nine of these boxes contained colonies that were found too numerous to count by either the primary investigator or the laboratory or both. Figure 6 shows the magnitude of the differences between the colony counts that the primary investigator and the laboratory obtained for the 14 and 31 boxes where the aerobic bacterial and fungal colony counts were not too numerous to count. Figure 6 shows that the absolute value of the differences were never larger than 4 and mostly below 2.

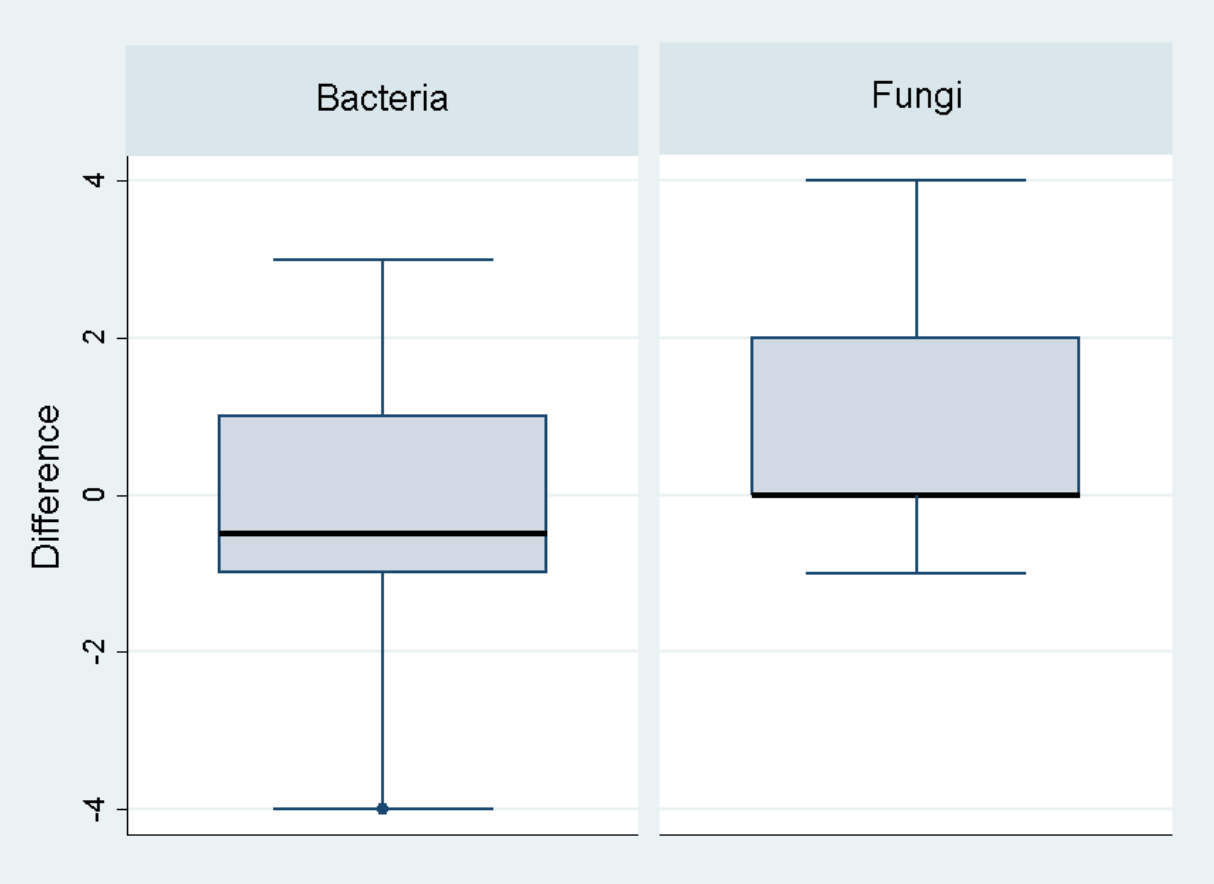

Figure 6: Difference between the colony counts that the primary investigator and the laboratory obtained in 14 and 31 incubation boxes for aerobic bacteria and fungi, respectively

(Boxes show the medians (bold lines) and interquartile ranges and the bars show the minima and maxima).

Table 5 summarises the counts obtained in the 14 boxes that both, the primary investigator and the laboratory counted and where neither found the colonies to be too numerous to count. There is no support for rejecting the null hypothesis that the counts obtained by the primary investigator and the laboratory were the same (P = 0.89).

Table 5: Summary of aerobic colony counts recorded by the primary investigator and the laboratory in the same 14 incubation boxes

	Primary investigator	Laboratory
Number of boxes	14	14
Mean	10.64	10.71
Standard error	1.91	1.85
Standard deviation	7.13	6.95
95% confidence of interval	6.52–14.76	6.70–14.72

Table 6 summarises the counts obtained in the 31 boxes that both, the primary investigator and the laboratory counted and where neither found the colonies to be too numerous to count.

There is support for the rejection of the null hypothesis of no difference between the counts obtained by the primary investigator and laboratory ($P = 0.01$). Table 6 shows that, on average, the primary investigator found 0.65 more fungal colonies than the laboratory did.

Table 6: Summary of fungal colony counts recorded by the primary investigator and the laboratory in the same 31 incubation boxes

	Primary investigator	Laboratory
Observations	31	31
Mean	6.61	5.96
Standard error	0.78	0.76
Standard deviation	4.32	4.25
95% confidence of interval	5.02–8.20	4.41–7.53

Because the primary investigator counted the microbial colonies in more boxes than the laboratory personnel did, and because their counts were similar with respect to aerobic bacterial colonies, and because the differences in their fungal counts were small, it was decided to only use the counts of the primary investigator for all further analyses.

4.2.2 Effect of Count category of aerobic bacterial and fungal colonies before boxes received vermiculite and eggs on Perhapsloststatus

Table 16 shows that only 2 boxes yielded no aerobic colonies and only 8 boxes yielded no fungi on the agar plates made before the boxes received vermiculite or eggs. Tables 7 and 8 show the frequency of the test boxes in the five Count categories of aerobic bacteria and fungi used in Study Main 1. These tables also show the range in the number of colonies in each Count category.

Table 7: Frequency of boxes in aerobic bacterial count categories in 100 incubation boxes before they received vermiculite and eggs, and the range of colonies found in each category

		Number of aerobic bacterial colonies		
Count category	Number of boxes	Minimum	Median	Maximum
0	12	0	2	5
1	18	6	7	13
2	14	14	16	20
3	16	21	26	60
4*	40			

* Boxes where the colony counts were TNTC.

Table 8: Frequency of boxes in fungal count categories in 100 incubation boxes before they received vermiculite and eggs, and the range of colonies found in each category

Count category	Number of boxes	Number of fungal colonies		
		Minimum	Median	Maximum
0	13	0	0	1
1	20	2	3	5
2	17	6	7	9
3	18	10	12	20
4*	32			

* Boxes where the colony counts were TNTC.

Table 9 shows that, excluding unbanded eggs, only 186 of 1963 eggs (9.48%) in the 100 test boxes had a Perhapsloststatus of one. Only 133 of the 1963 eggs (6.78%) were banded, while 53 of the eggs (2.70%) were rotten.

Table 9: Number of eggs in 100 incubation boxes beloning to the different hatching categories

	Number of eggs
Unbanded	482
Perhapsloststatus = 0	1777
Hatchling survived	1683
Mortality	46
Cull	29
Dead in shell	19
Perhapsloststatus = 1	186
Banded	133
Rotten	53

Table 10 and Table 11 show the frequency of eggs falling in different categories of the variable Count category with respect to aerobic bacteria and fungi against the response variable (Perhapsloststatus) as zero or one.

Table 10: Number of eggs that were either banded or rotten (Perhapsloststatus = 1) or not (Perhapsloststatus = 0) in 100 incubation boxes with different count categories of aerobic bacteria

	Count category					
Perhapsloststatus	0	1	2	3	4	Total
0	207	335	229	357	649	1777
1	6	24	33	31	92	186

Table 11: Number of eggs that were either banded or rotten (Perhapsloststatus = 1) or not (Perhapsloststatus = 0) in 100 incubation boxes with different count categories of fungi

	Count category					
Perhapsloststatus	0	1	2	3	4	Total
0	290	399	320	350	418	1777
1	17	25	60	28	56	186

Table 12 shows that, when controlling for counter, eggs in boxes having too numerous to count aerobic bacterial colonies (Count category 4) tend to have a higher log odds (P = 0.06) of being banded or rotten (Perhapsloststatus = 1) than eggs in boxes having numbers of colonies ranging from zero to below the 25% percentile (Count category 0). (Although the coefficient was 1.43 its 95% CI included zero (-0.09–2.94); hence the P-value is slightly above 0.05). Counter is a significant confounder (P < 0.05).

Table 13 shows the same model as Table 12, expressed as odds ratios. Table 13 shows that, on average, the odds for eggs being banded or rotten (Perhapsloststatus = 1) in boxes with too

numerous to count aerobic bacterial colonies (Count category 4) was 4.2 (0.92–18.95) times higher than for eggs in boxes with colony counts between zero and the 25% percentile (Count category 0).

Table 12: Effect of Count category of aerobic bacterial colonies on Perhapsloststatus

Perhapsloststatus	Coefficient	Standard error	Z	p > |z|	95% Conf. Interval
Count category					
1	0.68	0.78	0.87	0.39	-0.86–2.22
2	0.95	0.76	1.26	0.21	-0.54–2.43
3	0.71	0.77	1.92	0.36	-0.80–2.21
4	1.43	0.78	1.85	0.06	-0.09–2.94
Counter	0.66	0.30	2.21	0.03	0.08–1.24

Table 13: Effect of Count category of aerobic bacterial colonies on perhapsloststatus, expressed as odd ratios

Perhapsloststatus	Odds ratio	Standard error	Z	p > |z|	95% Conf. Interval
Count category					
1	1.98	1.55	0.87	0.39	0.42–9.20
2	2.57	1.95	1.25	0.21	0.58–11.35
3	2.03	1.56	0.92	0.36	0.45–9.13
4	4.17	3.22	1.85	0.06	0.92–18.95
Counter	1.93	0.30	2.21	0.03	0.08–1.24

Table 14 shows that the log odds of eggs being banded or rotten (Perhapsloststatus = 1) of boxes with fungal colony counts falling in any of count categories 1 to 4 did not differ from zero (P > 0.18) and thereby also not differ from the log odds of eggs being banded or rotten in boxes with fungal colony counts falling in Count category 0.

Table 15 shows that the odds of eggs being banded or rotten (Perhapsloststatus = 1) in boxes with fungal colony counts falling in any count categories 1 to 4 were the same as the odds of eggs being banded or rotten in boxes with fungal colony counts falling in Count category 0.

Table 14: Effect of Count category of fungal colonies on Perhapsloststatus

Perhapsloststatus	Coefficient	Standard error	Z	p > |z|	95% Conf. Interval
Count category					
1	-0.43	0.60	-0.73	0.47	-1.62–0.74
2	0.84	0.63	1.33	0.18	-0.40–2.08
3	-0.07	0.58	-0.12	0.91	-1.20–1.07
4	0.29	0.60	0.48	0.63	-0.90–1.47
counter	0.36	0.40	0.91	0.36	-0.42–1.15

Table 15: Effect of Count category of fungal colonies on Perhapsloststatus, expressed as odd ratios

Perhapsloststatus	Odds Ratio	Standard error	Z	p > |z|	95% Conf. Interval
Count category					
1	0.64	0.39	-0.73	0.47	0.20–2.10
2	2.32	1.47	1.33	0.18	0.67–8.01
3	0.93	0.54	-0.12	0.91	0.30–2.91
4	1.33	0.80	0.48	0.63	0.41–4.34
counter	1.43	0.58	0.91	0.36	0.65–3.16

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Table 16: Bacterial and fungal colony counts before the incubation period and the numbers of eggs that proofed to be banded or rotten (Perhaps lost) or unbanded after incubation in each of 100 incubation boxes

	Bacterial count 48 h		Fungal count 72 h		Fungal count 1 month	Eggs in the box (n)		
Box no.	Primary investigator	Laboratory	Primary investigator	Laboratory		Banded or rotten	Unbanded	Total
1 (9)[a]	2[*b]		0[*]		1	0	6	19
2 (10)	1[*]		0[*]		0	2	19	21
3 (11)	36[*]		7[*]		10	0	0	22
4 (12)	TNTC[c*]		15[*]		12[d]	0	6	23
5 (13)	TNTC[*]		12[*]		3	0	2	27
6 (14)	TNTC[*]		TNTC[*]		1	0	7	22
7 (15)	25[*]		9[*]		6	0	1	26
8 (16)	TNTC[*]		5[*]		5	2	4	23
9 (17)	TNTC[*]		TNTC[*]		TNTC	0	2	2
10 (18)	7[*]		2[*]		5	0	0	14
11 (19)	TNTC[*]		TNTC[*]		7	4	6	29
12 (20)	TNTC[*]		7[*]		0	1	0	19
13 (21)	0[*]		0[*]		0	0	1	14
14 (22)	TNTC[*]		10[*]		TNTC	0	22	27
15 (23)	24[*]		2[*]		2	3	12	36
16 (24)	TNTC[*]		0[*]		0	0	3	37
17 (25)	60[*]		3[*]		3	2	1	20
18 (26)	32[*]		7[*]		4	9	1	31
19 (27)	29[*]		1[*]		1	5	15	38
20 (28)	TNTC[*]		4[*]		4	1	1	20
21 (29)	16[*]		2[*]		5	1	0	19
22 (30)	TNTC[*]		0[*]		2	5	25	36
23 (31)	TNTC[*]		6[*]		TNTC	12	4	22
24 (32)	23[*]		1[*]		1	0	2	38
25 (33)	22[*]		10[*]		2	0	5	45
26 (34)	TNTC[*]		3[*]		1	5	0	29

Box no.	Bacterial count 48 h		Fungal count 72 h		Fungal count 1 month	Eggs in the box (n)		
	Primary investigator	Laboratory	Primary investigator	Laboratory		Banded or rotten	Unbanded	Total
27 (35)	TNTC*		10*		3	0	0	22
28 (36)	2*		0*		0	0	0	27
29 (37)	5*		2*		0	0	0	23
30 (38)	TNTC*		12*		9	3	0	19
31 (39)	TNTC*		TNTC*		6	2	20	23
32 (40)	26*		10*		6	0	3	38
33 (41)	TNTC*		TNTC*		TNTC	4	4	21
34 (42)	TNTC*		11*		1	6	8	32
35 (43)	TNTC*		9*		1	12	19	35
36 (44)	6*		1*		1	0	0	31
37 (45)	2*		1*		1	2	9	32
38 (46)	7*		0*		0	2	0	39
39 (47)	11*		0*	0	2	1	0	23
40 (48)	16*		7*	5	5	1	5	20
41 (49)	TNTC*		3*	3	4	0	15	27
42 (50)	26*		4*	2	5	1	2	34
43 (51)	0*		10*	8	8	1	10	36
44 (52)	TNTC*		11*	7	10	1	2	21
45 (53)	20*		9*	7	10	9	5	36
46 (54)	17*	TNTC	2*	1	4	0	0	22
47 (55)	21*	TNTC	7*	7	7	2	2	25
48 (57)	TNTC*	TNTC	3*	0	3	7	15	38
49 (58)	TNTC*	TNTC	4*	4	5	1	0	22
50 (59)	16*	17	5*	1	3	2	0	36
51 (60)	6*	4	6*	6	6	0	1	38
52 (61)	4*	5	2*	2	2	0	29	29
53 (62)	8*	7	3*	2	4	0	1	30
54 (63)	7*	8	6*	6	6	0	1	19
55 (64)	TNTC*	TNTC	6*	6	6	5	6	18

Box no.	Bacterial count 48 h		Fungal count 72 h		Fungal count 1 month	Eggs in the box (n)		
	Primary investigator	Laboratory	Primary investigator	Laboratory		Banded or rotten	Unbanded	Total
56 (65)	10*	10	2*	2	4	0	3	18
57 (66)	30*	27	12*	13	13	3	7	24
58 (67)	TNTC*	TNTC	8*	8	8	0	2	19
59 (68)	TNTC*	TNTC	9*	9	9	0	0	39
60 (69)	2*	3	3*	3	3	0	1	35
61 (70)	TNTC*	TNTC	6*	6	11	5	10	20
62 (71)	TNTC*	TNTC	1*	2	2	0	5	37
63 (72)	TNTC*	TNTC	5*	6	9	0	9	24
64 (73)	TNTC*	TNTC	TNTC*	TNTC	TNTC	2	0	19
65 (74)	6*	7	12*	11	13	0	2	23
66 (75)	TNTC*	TNTC	TNTC*	TNTC	TNTC	0	0	18
67 (76)	12*	11	13*	13	13	2	2	20
68 (77)	13*	13	TNTC	20*	TNTC	2	18	23
69 (78)	TNTC*	TNTC	5*	6	8	0	3	21
70 (79)	TNTC*	TNTC	TNTC*	TNTC	TNTC	4	6	33
71 (80)	TNTC*	TNTC	TNTC*	TNTC	TNTC	2	19	24
72 (81)	10*	10	8*	9	9	4	0	32
73 (82)	7*	7	TNTC*	TNTC	TNTC	0	2	21
74 (83)	18*	18	15*	14	14	5	0	32
75 (84)	TNTC*	TNTC	TNTC*	TNTC	TNTC	1	1	35
76 (85)	TNTC*	TNTC	TNTC*	TNTC	TNTC	1	8	26
77 (86)	TNTC*	TNTC	TNTC*	TNTC	TNTC	0	3	25
78 (87)	TNTC*	TNTC	18*	16	TNTC	0	0	19
79 (202)		17*		TNTC*	TNTC	0	4	19
80 (203)		10*		TNTC*	TNTC	0	2	14
81 (204)		21*		TNTC*	TNTC	0	0	15
82 (205)		5*		14*	TNTC	1	4	23
83 (206)		14*		TNTC*	TNTC	4	2	18
84 (207)		14*		TNTC*	TNTC	0	26	28

Box no.	Bacterial count 48 h		Fungal count 72 h		Fungal count 1 month	Eggs in the box (n)		
	Primary investigator	Laboratory	Primary investigator	Laboratory		Banded or rotten	Unbanded	Total
85 (208)		16*		TNTC*	TNTC	4	0	14
86 (209)		4*		9*	13	0	2	18
87 (210)		7*		TNTC*	TNTC	1	0	10
88 (211)		14*		TNTC*	TNTC	0	5	17
89 (212)		TNTC*		TNTC*	TNTC	3	4	13
90 (213)		7*		TNTC*	TNTC	6	0	14
91 (214)		28*		TNTC*	TNTC	2	0	16
92 (215)		24*		TNTC*	TNTC	2	4	17
93 (216)		20*		TNTC*	TNTC	4	9	17
94 (217)		15*		TNTC*	TNTC	1	2	20
95 (218)		TNTC*		TNTC*	TNTC	3	0	16
96 (219)		15*		TNTC*	TNTC	2	0	22
97 (221)		9*		TNTC*	TNTC	2	5	12
98 (222)		26*		TNTC*	TNTC	2	3	21
99 (223)		5*		TNTC*	TNTC	0	2	19
100 (224)		6*		17*	TNTC	4	5	20

[a] The number preceding the parenthesis is the sequential number of the test box used in the study, while the number in parenthesis is the sequential number of the clutch laid on the farm during the season.

[b] The asterisk indicates the count that was used for statistical analysis.

[c] TNTC = Too numerous to count

[d] A substantial decline in the number of fungal colonies sometimes occurred between 72 h and one month due to coalescence of neighbouring colonies, or overgrowth by one or a few colonies.

4.2.3 Difference in the proportion of TNTC colony counts before incubation and after hatching

The proportion of boxes that had too numerous to count aerobic bacteria increased during the incubation of the eggs ($P < 0.05$, Table 17). Similarly, the proportion of boxes that had too numerous to count fungal colonies also increased during incubation ($P < 0.05$, Table 17).

Table 17: Numbers of boxes with colony counts that were countable or too numerous to count (TNTC) before the boxes were loaded with vermiculite and eggs and after the boxes with eggs were incubated and the eggs have hatched

	TNTC	Countable
Aerobic bacteria after 48 h of incubation at 35 °C		
Before the boxes received eggs and incubation started	40[a]	60
After the boxes were incubated and the eggs hatched	98[a]	2
Fungi after 72 h of incubation at 25 °C		
Before the boxes received eggs and incubation started	32[b]	68
After the boxes were incubated and the eggs hatched	78[b]	22

[a, b] Proportions with the same superscript differ ($P < 0.05$)

Table 18 shows the colony counts of aerobic bacteria and fungi after the eggs were incubated and hatched.

Table 18: Aerobic bacterial and fungal colony counts after the incubation period and hatching of the eggs

Box No	Bacterial count 48 h	Fungal count 72 h	Fungal count 1 month
1(9)[a]	TNTC[b]	TNTC	TNTC
2(10)	TNTC	TNTC	TNTC
3(11)	TNTC	TNTC	TNTC
4(12)	TNTC	TNTC	TNTC
5(13)	TNTC	TNTC	TNTC
6(14)	TNTC	TNTC	TNTC
7(15)	TNTC	TNTC	TNTC
8(16)	11	11	TNTC
9(17)	TNTC	TNTC	TNTC
10(18)	TNTC	11	TNTC
11(19)	TNTC	TNTC	TNTC
12(20)	TNTC	TNTC	TNTC
13(21)	TNTC	TNTC	TNTC
14(22)	TNTC	20	TNTC
15(23)	TNTC	13	TNTC
16(24)	TNTC	0	0
17(25)	TNTC	TNTC	TNTC
18(26)	TNTC	38	TNTC
19(27)	TNTC	TNTC	TNTC
20(28)	TNTC	TNTC	TNTC
21(29)	TNTC	TNTC	TNTC
22(30)	TNTC	13	TNTC
23(31)	TNTC	TNTC	TNTC
24(32)	TNTC	TNTC	TNTC
25(33)	TNTC	TNTC	TNTC
26(34)	TNTC	TNTC	TNTC
27(35)	TNTC	TNTC	TNTC
28(36)	TNTC	TNTC	TNTC
29(37)	TNTC	TNTC	TNTC
30(38)	TNTC	TNTC	TNTC
31(39)	TNTC	TNTC	TNTC
32(40)	TNTC	TNTC	TNTC
33(41)	TNTC	TNTC	TNTC
34(42)	TNTC	31	TNTC
35(43)	TNTC	TNTC	TNTC
36(44)	TNTC	TNTC	TNTC
37(45)	TNTC	TNTC	TNTC
38(46)	TNTC	TNTC	TNTC
39(47)	TNTC	TNTC	TNTC
40(48)	TNTC	TNTC	TNTC
41(49)	TNTC	TNTC	TNTC
42(50)	TNTC	TNTC	TNTC

Box No	Bacterial count 48 h	Fungal count 72 h	Fungal count 1 month
43(51)	TNTC	TNTC	TNTC
44(52)	TNTC	TNTC	TNTC
45(53)	TNTC	14	TNTC
46(54)	TNTC	8	TNTC
47(55)	TNTC	TNTC	TNTC
48(57)	TNTC	14	TNTC
49(58)	TNTC	10	TNTC
50(59)	TNTC	3	TNTC
51(60)	TNTC	TNTC	TNTC
52(61)	TNTC	TNTC	TNTC
53(62)	TNTC	TNTC	TNTC
54(63)	TNTC	TNTC	TNTC
55(64)	TNTC	30	TNTC
56(65)	TNTC	TNTC	TNTC
57(66)	TNTC	TNTC	TNTC
58(67)	TNTC	TNTC	TNTC
59(68)	TNTC	0	0
60(69)	TNTC	TNTC	TNTC
61(70)	TNTC	TNTC	TNTC
62(71)	TNTC	TNTC	TNTC
63(72)	TNTC	19	TNTC
64(73)	TNTC	1	1
65(74)	TNTC	TNTC	TNTC
66(75)	TNTC	TNTC	TNTC
67(76)	TNTC	10	TNTC
68(77)	TNTC	TNTC	TNTC
69(78)	TNTC	TNTC	TNTC
70(79)	TNTC	TNTC	TNTC
71(80)	TNTC	TNTC	TNTC
72(81)	TNTC	TNTC	TNTC
73(82)	TNTC	TNTC	TNTC
74(83)	TNTC	TNTC	TNTC
75(84)	TNTC	TNTC	TNTC
76(85)	TNTC	TNTC	TNTC
77(86)	TNTC	TNTC	TNTC
78(87)	TNTC	TNTC	TNTC
79(202)	TNTC	TNTC	TNTC
80(203)	TNTC	4	TNTC
81(204)	TNTC	TNTC	TNTC
82(205)	TNTC	TNTC	TNTC
83(206)	TNTC	TNTC	TNTC
84(207)	TNTC	TNTC	TNTC
84(208)	TNTC	TNTC	TNTC
85(209)	TNTC	TNTC	TNTC
87(210)	TNTC	TNTC	TNTC

Box No	Bacterial count 48 h	Fungal count 72 h	Fungal count 1 month
88(211)	TNTC	TNTC	TNTC
89(212)	TNTC	TNTC	TNTC
90(213)	TNTC	TNTC	TNTC
91(214)	TNTC	8	TNTC
92(215)	TNTC	TNTC	TNTC
93(216)	TNTC	TNTC	TNTC
94(217)	TNTC	TNTC	TNTC
95(218)	TNTC	20	TNTC
96(219)	TNTC	TNTC	TNTC
97(221)	TNTC	TNTC	TNTC
98(222)	TNTC	TNTC	TNTC
99(223)	TNTC	TNTC	TNTC
100(224)	0	0	TNTC

[a] The number preceding the parenthesis is the sequential number of the test box used in the study, while the number in parenthesis is the sequential number of the clutch laid on the farm during the season.

[b] TNTC = Too numerous to count

4.3 Species of aerobic bacteria, anaerobic bacteria and fungi isolated from unhatched eggs

Table 19 and 20 show the species of aerobic bacteria, anaerobic bacteria and fungi isolated from eggs that failed to hatch in the 10 clutches with the lowest- and highest hatching percentages, respectively.

Table 19: Species isolated from the 10 boxes with the lowest hatching percentages

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
1(10)[a]	1	*Citrobacter koseri* *Comamonas testeroni*		*Fusarium species*
	2	*Citrobacter koseri*		*Cladosporium species*
	3			*Cladosporium species*
2(22)	1	*Citrobacter koseri*		
	2	*Citrobacter koseri* *Achromobacter xylosoxidans* *Escherichia coli*		*Cladosporium species*
	3	*Enterobacter cloacae* *Sphingobacterium spiritivorum*	*Veillonella species*	*Cladosporium species*
3(29)	1	*Citrobacter koseri* *Achromobacter xylosoxidans*		*Fusarium species*
	2	*Citrobacter koseri* *Achromobacter xylosoxidans*		
4(30)	1	*Escherichia coli* *Sphingomonas paucimobilis*		*Fusarium species*

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
	2	*Citrobacter koseri* *Escherichia coli*		*Cladosporium species*
5(39)	1	*Achromobacter xylosoxidans* *Enterobacter cloacae*	*Veillonella species*	
	2	*Enterobacter cloacae*		
6(43)	1	*Citrobacter koseri*		
	2	*Citrobacter koseri* *Escherichia coli* *Sphingomonas paucimobilis* *Pseudomonas aeruginosa*	*Veillonella species*	*Cladosporium species*
7(61)	1	*Citrobacter koseri*		
	2	*Citrobacter koseri* *Enterobacter cloacae* *Sphingomonas paucimobilis* *Pseudomonas stutzeri*		
8(77)	1	*Escherichia coli*		
	2	*Citrobacter koseri* *Enterobacter cloacae* *Sphingomonas paucimobilis* *Pseudomonas aeruginosa*		*Penicillium species*

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
9(80)	1	*Citrobacter koseri* *Sphingomonas paucimobilis* *Burkholderia cepacia*		*Fusarium species*
	2	*Enterobacter cloacae* *Sphingomonas paucimobilis* *Serratia odorifera*		*Fusarium species*
10(207)	1	*Enterobacter cloacae*		*Fusarium species*
	2	*Comamonas testeroni* *Escherichia coli*		
	3	*Citrobacter koseri*		*Fusarium species*
	4	*Enterobacter cloacae*		*Paecilomyces species*
	5	*Citrobacter koseri*	*Clostridium sporongenes*	*Fusarium species*

[a] The number preceding the parenthesis is the sequential number of the test box used in the study, while the number in parenthesis is the sequential number of the clutch laid on the farm during the season.

Table 20: Species isolated from the 10 boxes with the highest hatching percentages

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
1(13)[a]	1	*Citrobacter koseri* *Achromobacter xylosoxidans*		*Cladosporium species*
	2	*Citrobacter koseri* *Escherichia coli*		*Cladosporium species* *Aspergillus versicolor*
2(20)	1	*Citrobacter koseri* *Enterobacter cloacae*		
	2	*Citrobacter koseri* *Achromobacter xylosoxidans* *Enterobacter cloacae*		
3(24)	1	*Citrobacter koseri* *Achromobacter xylosoxidans*		*Cladosporium species*
	2	*Citrobacter koseri*		*Cladosporium species*
	3	*Citrobacter koseri* *Comamonas testeroni*		
4(28)	1	*Escherichia coli*		*Fusarium species*
	2	*Citrobacter koseri*		

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
5(32)	1	*Citrobacter koseri* *Achromobacter xylosoxidans* *Escherichia coli* *Pseudomonas aeruginosa*		*Fusarium species*
	2	*Citrobacter koseri* *Escherichia coli* *Sphingomonas paucimobilis* *Burkholderia cepacia*		
6(35)	1	*Citrobacter koseri*		
	2	*Citrobacter koseri*		*Penicillium species*
7(40)	1	*Citrobacter koseri* *Achromobacter xylosoxidans* *Sphingomonas paucimobilis*		
	2	*Escherichia coli* *Sphingomonas paucimobilis*		*Fusarium species* *Cladosporium species*
8(46)	1	*Enterobacter cloacae* *Sphingomonas paucimobilis*		*Fusarium species*
	2	*Citrobacter koseri* *Achromobacter xylosoxidans* *Escherichia coli*		*Fusarium species*

Box number	Egg number	Aerobic bacteria	Anaerobic bacteria	Fungi
9(59)	1	*Enterobacter cloacae*		*Fusarium species*
	2	*Citrobacter koseri* *Achromobacter xylosoxidans* *Escherichia coli*		
10(82)	1		*Veillonella species*	
	2	*Citrobacter koseri*		

[a] The number preceding the parenthesis is the sequential number of the test box used in the study, while the number in parenthesis is the sequential number of the clutch laid on the farm during the season.

The most frequently occurring species of aerobic bacteria, anaerobic bacteria and fungi in the lowest hatching percentage group also had the highest frequency in the highest hatching percentage group (Table 21).

Table 21 shows that no two unhatched eggs from any of the 10 boxes with the lowest hatching percentages contained any aerobic, anaerobic or fungal species that did not also occur in at least one unhatched egg from at least one of the 10 boxes with the highest hatching percentages. There were 3 aerobic and one anaerobic species of bacteria and one species of fungi that were present in one but not both the unhatched eggs from at least one of the 10 clutches with the lowest hatching percentages that were absent in the unhatched eggs from the clutches with the highest hatching percentages. There was one species of fungi present in one unhatched egg from one of the 10 clutches with the highest hatching percentages that was absent in the unhatched eggs from the clutches with the lowest hatching percentages.

Table 21: Frequency of microbial species isolated from unhatched eggs in the incubation boxes with the lowest and highest hatching percentage boxes (10 boxes per group)

	Number of boxes	
Aerobic bacteria	Lowest hatching percentage	Highest hatching percentage
Citrobacter koseri	9	10
Enterobacter cloacae	6	3
Escherichia coli	5	6
Sphingomonas paucimobilis	5	3
Achromobacter xylosoxidans	3	7
Comamonas testeroni	2	1*
Pseudomonas aeruginosa	2	1*
Burkholderia cepacia	1*	1*
Pseudomonas stutzeri	1*	
Serratia odorifera	1*	
Sphingobacterium spiritivorum	1*	
Anaerobic bacteria		
Veillonella spp.	3	1*
Clostridium sporongenes	1*	
Fungi		
Fusarium spp.	5	5
Cladosporium spp.	4	3
Paecilomyces spp.	1*	
Penicillium spp.	1*	1*
Aspergillus versicolor		1*

* Isolated from only one egg of a clutch.

Chapter 5
Discussion

5.1 The main findings of the study

The primary aim of this study was to determine whether eggs in boxes that had more aerobic bacterial colonies and more fungal colonies before they received vermiculite and eggs are more prone to fail to develop to term than eggs in boxes that had fewer colonies before they received vermiculite and eggs. The current study shows that the odds of eggs failing to develop to term in boxes that had too numerous to count aerobic bacterial colonies tended to be higher than the odds of eggs in boxes that had 5 or fewer aerobic bacterial colonies. The current study also shows that the number of fungal colonies in boxes before they were loaded with vermiculite and eggs had no effect on the odds of failing to develop to term.

The second aim was to determine whether there are species of aerobic bacteria, anaerobic bacteria and fungi that are present in unhatched eggs from clutches with low hatching percentage but not in clutches with high hatching percentage without there being microbial species that occur in boxes with high hatching proportions that do not also occur in unhatched eggs from boxes with low hatching proportions. The current study shows no species of aerobic bacteria, anaerobic bacteria or fungi that occurred in each of at least two unhatched eggs from at least one box with a low hatching percentage that did not also occur in at least one egg from at least one of the boxes with high hatching percentages. Similarly, no bacterial or fungal species were isolated from each of at least two unhatched eggs from boxes with high hatching proportions that did not also occur in unhatched eggs from boxes with low hatching proportions.

5.2 From where in incubation boxes should plate agar impressions be taken?

The aim of the pilot study was to determine the preferred site from which to obtain agar plate impressions during the main study. The pilot study showed that the microbial load (aerobic bacterial and fungal colonies) differ among the centre of the under surface of the lid, the centre of the floor, near the bottom corner of two sides and the centre of a wall on the inside of the incubation box. The centre of the floor had higher aerobic bacterial and fungal colony counts than the underside of the lid and tended to have more aerobic bacterial colonies than near the bottom corner of two sides. Hence the selection of centre of the floor as the preferred site from which to obtain plate agar impressions for the aerobic bacterial and fungal colony counts in Study Main 1 was justified.

The centre of the floor yielded similar numbers of colonies than near the bottom corner of two sides in the case of aerobic bacteria and the centre of a side wall in the case of both, aerobic bacteria and fungi. One may therefore also have collected the contact agar plate samples from these sites but due to cost constraints it was decided to limit the sampling to the centre of the floor for aerobic bacteria and fungi.

5.3 Study Main 1

5.3.1 The effect of different persons having counted the microbial colonies

A shortcoming of this study is that, for some boxes, different persons (the Primary investigator and the Laboratory technologist) counted the aerobic bacterial colonies and the fungal colonies

cultured from the incubation boxes before they received vermiculite and eggs. Although it was preferred that the laboratory personnel would do the counts, they would not have been available over the Christmas holiday season, which necessitated the primary investigator to do some counts on his own. There were a number of boxes where both, the primary investigator and the laboratory personnel counted the colonies. Comparing these counts showed that the primary investigator and the laboratory personnel obtained similar numbers of aerobic bacterial counts and that the primary investigator, on average, found about 0.65 more fungal colonies than the Laboratory did. Numerically, the differences between fungal counts obtained by the laboratory personnel and the primary investigator were small (never larger than four and mostly smaller than two). The primary investigator counted the microbial colonies in more boxes than the laboratory did. In the light of the above the decision to use only the counts of the primary investigator is justified.

When controlling for the number of aerobic bacterial colonies (Count category), the odds of eggs being banded or rotten was 1.93 times higher when the counts of the laboratory were considered compared to when those of the primary investigator were considered ($P < 0.05$). The person doing the colony counts was a significant confounder for the effect of aerobic bacterial colony counts on the odds of eggs being banded or rotten and its inclusion in the model as a second independent variable was therefore justified.

Although also included in the model to assess the effect of fungal colony counts on the odds of eggs being banded or rotten, the person doing the counts had no significant effect.

5.3.2 Effect of the numbers of aerobic bacterial- and fungal colonies on the odds of eggs being banded or rotten

Only 9.5% of eggs had a Perhapsloststatus of one, including 6.8% that were banded and 2.7% that were rotten. It is not known what proportion of the rotten eggs were actually embryonated or not. Overall at most 6.8% of the eggs were banded may therefore have lost a viable conceptus. In Study Main 1 we focused on this 9.5%, attempting to relate the odds of their occurrence to microbial colony counts in the incubation boxes.

Ninety eight per cent of incubation boxes yielded at least one colony of aerobic bacteria and 92% yielded at least one fungal colony on the agar plate impressions collected before they received vermiculite and eggs. This is remarkable because the boxes had been washed and disinfected repeatedly before the agar plate impressions were done. Studies have shown bacterial resistance to disinfectants used in poultry hatcheries (Willingham et al. 1996; Qureshi 2002). It is not known whether the aerobic bacterial colonies and the fungal colonies represent species that are resistant to the disinfectants or not.

This study shows that, controlling for counter, the odds of eggs being banded or rotten is expected to be 4.2 times higher in boxes that had too numerous to count aerobic bacterial colonies before they received vermiculite or eggs than in boxes that had between zero and 5 aerobic bacterial colonies. The 95% confidence interval (0.92–18.95) does, however, include one. The P value of 0.06 for this comparison shows sufficient support for the null hypothesis of the odds being equal at the 5% level of significance. At a 6% level of significance, the confidence interval would not include one, providing sufficient support for the rejection of the null hypothesis. When the odds of eggs being banded or rotten in boxes with fewer than too

numerous to count aerobic colonies (Count categories 1, 2 and 3) were respectively compared to the odds of eggs being banded or rotten in the group of boxes with 0–5 aerobic colonies (Count category 0), there was insufficient support at the 5% level of significance to reject the null hypothesis that the odds of the two groups being compared are equal.

At the 5% level of significance, the odds of eggs being banded or rotten in boxes that had too numerous to count fungal colonies before they received vermiculite and eggs was equal to the odds of eggs being banded or rotten in boxes that had between zero and one fungal colonies. Similarly, at the 5% level of significance, the odds of eggs being banded or rotten from the boxes having fewer fungal colonies than too numerous to count (10 to 20, as represented by Count category 3) were the same as the odds for eggs in boxes that had between zero and one fungal colonies.

5.3.3 Fungal count after one month of incubation

Colony count for fungi after one month was done to count slow–growing fungi. The fungal counts after one month were sometimes substantially lower than the counts after 72 h. This decline was presumably due to coalescence of neighbouring colonies, or overgrowth by one or a few fungal colonies. Hence the counts after one month was not included in the statistical analysis.

5.3.4 Aerobic bacterial and fungal colony counts after the hatching period

The results of colony counts of bacteria and fungi from the test boxes after the incubation period showed that there was significant increase ($P < 0.001$) in the proportion of boxes with TNTC colonies. Only 2% of the boxes did not have TNTC aerobic bacterial colonies and only 22% of

the boxes did not have TNTC fungal colonies after the hatching period as compared to 60% and 68% of the boxes before they were loaded with vermiculite and eggs.

This significant increase of colony counts may be due to contamination from various sources, such as the following: The contamination that was present in the boxes before they received vermiculite and eggs; the incubator, especially because it has high humidity and favourable temperature that may sustain microbial growth; the vermiculite and the eggs themselves.

5.4 Study Main 2

In poultry, species of bacteria are associated with egg shell penetration ultimately poor hatching percentage (Higenyi and Kabasa 2014). De Reu et al. (2006) studied bacterial penetration through the egg shell, shell membrane and albumen into the yolk of chicken eggs and found penetration of *Staphylococcus warneri*, *Acinetobacter baumannii*, *Alcaligenes* spp., *Serratia marcescens*, *Carnobacterium* spp., *Pseudomonas* spp., and *Samonella enteritidis*.

Species of aerobic bacteria, anaerobic bacteria and fungi were isolated from unhatched eggs in the lowest and highest hatching percentage clutches. The variety and distribution of microbial species isolated from unhatched eggs of lowest hatching and highest hatching clutches respectively, was almost same. *Pseudomonas stutzeri, Serratia odorifera* and *Sphingobacterium spiritivorum* were isolated only from lowest hatching percentage clutches, but from only one egg of a clutch. There was no such aerobic bacterial species which was isolated from any egg of a clutch of highest hatching percentage but did not isolated from any egg of any clutch of lowest hatching percentage.

Clostridium sporongenes was the only anaerobic bacterial species which was isolated only from one egg of a clutch of lowest hatching percentage and was not isolated from any egg of any clutch of highest hatching percentage.

Paecilomyces spp. was the only fungal species which was isolated only from one egg of a clutch of lowest hatching percentage, while *Aspergillus versicolor* was the only fungal species which was isolated only from one egg of a clutch of highest hatching percentage.

The pattern of species isolated from unhatched eggs suggests that they are more likely pertaining to contamination from the cloaca of female crocodile (as cloaca might be contaminated by soil easily) or from the environment i.e. nesting material, personnel handling, vermiculite, incubation box or the incubator environment. Most of the species isolated in our study are normally present in environment, or body of animals and humans and opportunistic pathogens. *Citrobacter* spp. are part of the normal enteric flora of reptiles (Jacobson 2007), other animals and humans (Nayar et al. 2014). *Citrobacter* spp. are opportunistic causes of enteritis in humans (McVey et al 2013) and have been isolated from chorioallantois of alligator eggs (Schumacher and Cardeilhac 1990). *Enterobacter cloacae is* normally present in soil, water, intestines of humans and animals (Liu et al. 2013; Markey et al. 2013) and is an opportunistic pathogen causes urogenital tract infections in animals (McVey et al 2013). *Escherichia coli* is an important pathogen of animals (Hirsh et al. 2004; McVey et al 2013), and a normal inhabitant of the large and small intestines of animals. *Escherichia coli* also causes omphalitis in chicks (Markey et al. 2013; McVey et al 2013). *Escherichia coli* has also been isolated from intestinal flora of wild Nile crocodiles (Lovely and Leslie 2008), from frozen Nile crocodile meat (Makanyanga et al. 2014), and the shell membranes of unhatched crocodile eggs (Foggin 1992). *Sphingomonas paucimobilis* is widely spread in the environment, including water (Murray et al. 1995). *Achromobacter* spp. are

frequently present in the environment and are opportunistic pathogens in animals (Markey et al. 2013). Most of the strains of *Pseudomonas* live in soil and water (McVey et al 2013; Markey et al. 2013). *Pseudomonas* spp. are often resistant to antimicrobials (Markey et al. 2013). *Pseudomonas* spp. have been isolated from shell membranes of unhatched crocodile eggs (Foggin 1992), and the chorioallantois of alligator eggs (Schumacher and Cardeilhac 1990). *Burkholderia* spp. occur in the environment and are distributed worldwide (McVey et al 2013; Murray et al. 1995). *Clostridium* spp. are normally present in soil and fresh water (Quinn et al. 2002).

Fungi can grow at wide range of organic matter and are distributed worldwide (Markey et al. 2013). *Fusarium* spp. are soil saprophytes and may cause infections in animals and humans (Murray et al. 1995). *Fusarium* spp. have been isolated from liver, lungs, small intestine and bowel samples of infected crocodiles (Hibberd 1993), and from eggs of *Crocodylus porosus* and *Alligator mississippiensis* (Huchzermeyer 2003). *Paecilomyces* spp. are soil saprophytes and play significant role in biodeterioration (decomposition of organic compounds) (Murray et al. 1995), isolated from eggs of *Crocodylus porosus* and *Alligator mississippiensis* (Huchzermeyer 2003).

The swabs collected from at least two unhatched egg of each of the 10 lowest hatching percentage boxes and the 10 highest hatching percentage boxes were used to isolate aerobic bacteria, anaerobic bacteria and fungi, and could not be further used for the enrichment media of *Salmonella* isolation from each egg separately. We therefore pooled the swabs collected from at least two eggs of a clutch and used the pooled sample for the isolation of *Salmonella* spp. *Salmonella* spp. were isolated from the unhatched eggs of three clutches of lowest hatching percentage and four clutches of highest hatching percentage. The species isolated and identified

from lowest hatching percentage clutches are following: *Salmonella. Typhimurium* 4, 5:i:1,2, *S. blockley* 6, 8 : k: 1, 5, *S stachus* 38: z:-. The species of *Samonella* isolated and identified from the unhatched eggs of highest hatching percentage clutches are following: Salmonella III 35:k:z, *S. mango* 38 :k: 1, 5. *Salmonella poly* OE. Salmonella III 35:k:z. The percentage frequency of *Salmonella* spp from lowest hatching percentage and highest hatching percentage was almost same. So, we cannot say that *Salmonella* spp. are associated only with the lowest hatching percentage clutches.

We did not attempt to isolate and identify the species of aerobic bacteria, anaerobic bacteria and fungi from the incubation boxes or the nest soil or the vermiculite due to cost constrains. Comparing the isolates from those sources with the isolates from the eggs would shed more light on the origin of the species found in the eggs.

5.5 Future research

The current study was observational. Eggs from boxes that had too numerous to count aerobic bacterial colonies before they received vermiculite or eggs tended to have higher odds of being banded or rotten than the odds for eggs in boxes with zero to 5 colonies. This indicates that there may be an association between the odds of eggs being banded or rotten and the level of aerobic bacterial contamination present in incubation boxes before they receive vermiculite and eggs. We cannot conclude that the boxes with TNTC aerobic bacterial colonies caused the eggs to be banded or rotten more than the eggs in boxes with fewer colonies as an association does not indicate a causal effect. The following experiment design should enable one to better demonstrate a causal effect of the microbial load in incubation boxes before they receive eggs and the outcome of incubation and hatchery:

To find the effect of microbial contamination from the incubation boxes on the hatching percentage of eggs, one can find the microbial load from the source population of incubation boxes before the laying period and then store the incubation boxes under conditions avoiding any contamination until the boxes receive eggs for incubation. From the source population these incubation boxes should then be divided in two main groups of boxes. One then identify those with no or very low colony counts and designate them to group low. One may similarly identify and assign those boxes with TNTC colony counts to group high. Half of the eggs from the same clutch will be placed in one of the box from group low and the other half will be placed in one of the boxes from group high for further incubation under identical conditions.

The results of such kind of proposed study well demonstrate whether or not there is a causal relationship of microbial load and hatching percentage. If such a study fails to show a causal relationship between prior microbial load in incubation boxes and eventual hatching proportion, the research focus may have to shift to other possible causes, such as differences in immune capability among cluthces.

5.6 Final conclusions

This observational study shows that there is a trend of association of aerobic bacterial colony counts from the incubation boxes of Nile crocodile eggs (before they are loaded with vermiculite and eggs) and the eggs to become non-viable (foetus died during incubation) or become rotten. There is no association of fungal colony counts from the boxes before they are loaded with vermiculite and eggs and the eggs to become non–viable or rotten during incubation period. The other conclusion is that the species of aerobic bacteria, anaerobic bacteria and fungi isolated from unhatched eggs of lowest hatching percentage clutches and highest hatching percentage

clutches are almost similar and more likely pertaining to contamination from the environment i.e. nesting material of clutch, personnel handling, vermiculite, incubation box or the incubator environment.

Summary

The South African crocodile industry has the potential to produce Nile crocodile skins, which are in high demand by the global fashion industry. The crocodile industry is an important economic resource to South Africa and Southern Africa. The main purpose of rearing Nile crocodiles at intensive production units is to produce viable hatchlings which will be grown to produce quality skins. The production of sufficient hatchlings depends on high hatching percentage of clutches. It has been observed that the hatching percentage varies among clutches. We do not know that whether the poor hatching percentages are associated with the microbial load from the incubation boxes or not. The aims of the study were to determine whether eggs in boxes that had more aerobic bacterial colonies and more fungal colonies before they received vermiculite and eggs are more prone to become lost from the pool potentially capable of yielding conceptuses developing to term than eggs in boxes that had fewer colonies before they received vermiculite and eggs, and whether there are species of aerobic bacteria, anaerobic bacteria and fungi that are present in unhatched eggs from clutches with low hatching percentage but not in clutches with high hatching percentage without the converse being true.

At the time of hatching the farm personnel classify hatchlings or unhatched eggs into different categories e.g. hatchling survived, culled, foetus died in the shell, hatchling died, unfertilised egg, banded (the foetus died long before hatching), or rotten egg (putrefaction rendering the fertilisation status unknown). We assumed that banded or rotten eggs are more likely to have lost

conceptuses due to the microbial load that was present in the incubation boxes before they were loaded with vermiculite and eggs than the other classes of eggs. A response variable "Perhapsloststatus" was therefore created. For each egg that was either classified as Banded or Rotten Perhapsloststatus was assigned the value of one. For each egg belonging to other categories Perhapsloststatus was assigned the value of zero. Eggs classified as unfertilised were excluded from Perhapsloststatus.

The microbial load of aerobic bacteria and fungi from the incubation boxes before they were loaded with vermiculite and eggs was measured as colony forming units (CFU). The independent variable of interest was Count category (a categorical variable of aerobic bacteria and fungi colony counts), which was compiled as follows:

0 = Counts zero to below the 25th percentile, 1 = Counts from the 25th percentile to below the 50th percentile, 2 = Counts ranging the 50th percentile to below the 75th percentile, 3 = Counts from the 75th percentile to the maximum that was countable and 4 = Too numerous to count.

Sterile swabs were used to collect samples from unhatched eggs for isolation and identification of aerobic bacteria, anaerobic bacteria and fungi from the 10 boxes with the lowest and highest hatching percentages, respectively.

We used a population-averaged mixed-effect logistic regression model to determine the effect of Count category on Perhapsloststatus.

The odds for eggs being banded or rotten (Perhapsloststatus = 1) in boxes with too numerous to count aerobic bacterial colonies (Count category 4) tended to be higher than for eggs in boxes with colony counts between zero and the 25% percentile (Count category 0) (P = 0.06). The odds

of eggs being banded or rotten (Perhapsloststatus = 1) in boxes with fungal colony counts falling in any count categories 1 to 4 were the same as the odds of eggs being banded or rotten in boxes with fungal colony counts falling in Count category 0.

The variety and frequency of species of aerobic bacteria, anaerobic bacteria and fungi from unhatched eggs of lowest hatching boxes and highest hatching boxes was almost the same. The species isolated from unhatched eggs are more likely pertaining to contamination from the cloaca of female crocodile or from the environment i.e. nesting material, personnel handling, vermiculite, incubation box or the incubator environment, as most of the isolated species present are normally present in soil, water, dust and also opportunistic pathogens in animals.

This observational study shows that there is a trend of association between the aerobic bacterial load from the incubation boxes and the eggs in them to become either banded or rotten.

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