Bacterial and fungal penetration of the Nile crocodile (*Crocodylus niloticus*) egg in relation to the eggshell and eggshell membrane anatomy and microstructure

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Abstract

Commercial farming with the Nile crocodile (*Crocodylus niloticus)* for both leather and meat is an economically significant industry in South Africa. Due to the economic value of these products, effective captive breeding and hatchling rearing management programmes are of critical importance. An increase of knowledge and the investigation of specific crocodile industry related questions (identified interventions) in South Africa are of utmost importance to standardize and optimize Good Operating Practices for Nile crocodile husbandry, as well as to increase productivity on commercial farms. Low hatching rates have been identified as a management problem by most South African farms. Although many factors may contribute to the low hatching percentage, the role of bacterial and fungal contamination of the embryo inside the egg, has not been investigated before. For these microorganisms to clinically affect the healthy embryo, they must be able to gain access through the eggshell and the eggshell membrane. The role of the anatomy and microstructures of the eggshell in the prevention of intra-egg infections, and its changes during the incubation period is also not well known.

The Objectives of this project were to: (1) test, optimize and determine the most suitable preparation technique / set of techniques to comprehensively describe the microstructure of the Nile crocodile eggshell and eggshell membrane; (2) investigate and describe the normal anatomy of the Nile crocodile's eggshell and eggshell membranes and its changes during the incubation period; (3) identify the bacterial and fungal species prevalent on commercial crocodile farms and associated with the Nile crocodile egg after oviposition and during the incubation period; (4) investigate the penetration of bacterial and fungal species through the eggshell and membrane; and (5) determine if there is a correlation between the changing microstructure of the egg during incubation and the ability of microbes to penetrate the eggshell and eggshell membranes.

Fifty unbanded *Crocodylus niloticus* eggs from 22 clutches, 12 nesting sites on three different farms were used for Objective 1. A variety of sample preparation and analysis methodologies and techniques was tested and optimized to study the micro- and ultrastructure of the inorganic eggshell and its associated organic eggshell membrane.

For Objectives 2, 3 and 4, *Crocodylus niloticus* eggs (n = 48) from 12 clutches, each from different nesting sites on three separate farms were collected. Four eggs from different incubation stages (one day -, one week -, five weeks – and nine weeks after oviposition) were collected from each of the 12 clutches. During the first egg collection relevant environmental samples and samples from areas associated with the egg during incubation (nesting material, water, incubator boxes, vermiculite, and egg handlers' hands) were also collected for microorganism identification. In addition, any eggs (n =

17) that showed external signs of microbial infection (discoloration or rotting) or embryo death (egg cool to the touch) during the incubation period were identified and collected. Bacterial and fungal species were identified on all the collected samples and material using MALDI-TOF biomolecule analysis and ITS sequencing, respectively.

Following testing and optimization of numerous techniques and sample preparation approaches, a combination of light and electron microscopy techniques, and micro/nano computed tomography was selected to investigate and comprehensively describe the anatomical and morphological information of the Nile crocodile eggshell and eggshell membranes.

The *Crocodylus niloticus* egg is a translucent white ellipsoid egg and classified in the histostructural organization as a crocodyloid-type egg and comparative to similar species such as the American alligator. The shell is divided into three basic zones namely, the external, column / palisade and mammillary layers. The external layer is mainly composed of phosphorus and an amorphous form of calcite, the palisade layer of crystalline calcite arranged in large wedge-shaped shell units with traces of phosphorus, magnesium, sodium, and sulphur. The innermost mammillary layer is small irregular calcite crystals arranged in rosette-like structures in which the eggshell membrane fibres are embedded. The eggshell membrane is composed of several layers of organic fibre sheets arranged at right angles to one another, with a thin inner limiting membrane isolating the egg contents. Between the mammillary knobs and the membrane several airspaces exist which is connected to the pore channels running through the eggshell. Both the mammillary knobs and pore channels are found in higher densities at the equator of the egg reducing in number towards the egg poles; the shell is also at its thickest at the egg equator. During incubation, the mammillary knobs are the main source of calcium for the developing embryo, the calcium resorption can be seen as erosion and flattening of the mammillary knobs, thinning of the shell and a disruption in the shell-membrane connection. This movement of calcium is also believed to contribute to the colour change of the eggshell after fertilization and during incubation known as the banding phenomenon as higher amounts of calcium are found in the equatorial white area (banded) if compared to the unbanded area.

Molecular analysis of 48 healthy, 17 dead-in-shell or infected eggs and environmental samples identified over 100 different bacterial species from 35 different genera and 22 fungal species from 10 genera. Fifteen bacterial species and 4 fungal species were found to be able to penetrate through the eggshell membrane and comprise the integrity of the egg contents and developing embryo. The diversity of species identified on the eggshell surface and in the egg contents changed notably during the incubation period, with bacterial and fungal species ultimately decreasing on the eggshell surface and increasing in the egg contents. A third of the bacterial species identified (ex. *Aeromonas, Citrobacter, Escherichia, Proteus, Pseudomonas* and *Salmonella*) are known to be significant opportunistic pathogens and several have been implicated as causing reptile pathology and embryonic death in oviparous animals. At least 5 of the species of fungi found (ex. *Aspergillus, Fusarium* and

Lichtheimia) are also well-known causative agents of crocodile disease and a few are problematic in poultry farming as they are known to be able to infect the egg contents.

During the last collection period (± 9 weeks after oviposition), the diversity of bacterial and fungal species on the external shell surface increased sharply following a steady decline during the earlier stages of the incubation period. This corresponds to and might show a relationship with the constituent changes occurring in the external layer of the shell during the incubation period, i.e., the loss of phosphorus, which has been linked to reduced microbial defence and the entrance of bacteria in avian eggs. Further correlation between the changing ultrastructure of the eggshell and its membrane, and the diversity of microbes found in association with the egg is insubstantial. Elapsed time, the artificial incubation environment favouring specific species and weakening of the membrane due to bacterial presence are more likely to be the cause of increased bacterial and fungal species isolated from the eggshell content.

The eggshell and microstructure are very well adapted through evolution for the successful reproduction of amniotes. Significant first-time observations and contradictions to previous findings relating to the eggshell and membrane structures of the Nile crocodile egg, including the pattern of variable shell thickness, the existence of airspaces, the distribution and presence of mammillae and pores, and the external layers' crystal structure and composition were made. This information advances the academic knowledge on the subject area of *C. niloticus* reproductive biology and can also be used in future studies to determine the synthesis requirements and fundamental parameters of the shell which might lead to optimized procedures in basic husbandry and the artificial incubation environment (Standard Operating Procedures). It seems as if the anatomy of the eggshell and eggshell membrane is not sufficient to stop the penetration of bacteria and fungi, although it does impede and slow down the movement of microbes adequately to allow embryo development to continue to a point where the embryo and other extraembryonic elements are more resistant to the detrimental effects of microbial penetration. If bacterial and fungal agents are not delayed and allowed to penetrate the eggshell and membrane during early-stages of embryonic development, there is a high risk of infection and embryo mortality. Penetration into the egg contents by microbes during the later developmental stages is still associated with possible pathologies due to indirect causes such as oxygen deprivation, toxic stress, or egg water depletion.

High environmental microbial loads, egg handling and transport shortly after oviposition, and the introduction to shared artificial incubation spaces, as it is routinely practised in crocodile husbandry, introduce the collected eggs to numerous microorganisms. This exposure also occurs at a point during development where the egg is at its most susceptible to infection endangering the embryo, but also the commercial enterprise. The knowledge of the microbial diversity on farms, and in association with the time points or points where high loads of unnecessary microbes are introduced, will be able to be used to aid in specific interventions to reduce the microbial load, infection risks, embryo mortality and ultimately reduced hatchability. The knowledge of the specific organisms able to penetrate the physical

barriers of the egg and the species most often associated with embryo pathogenicity and / or mortality can also be applied to identify and select treatment protocols of the collected eggs directed to target the specifically implicated microorganisms.

Potential benefits of this PhD project

1. Knowledge of bacterial and fungal microorganisms directly affecting the Nile crocodile egg on South African crocodile farms
2. The microstructure of the eggshell and its membrane, and a possible correlation between the microanatomy and associated microbial infections would contribute significantly to the improvement of current husbandry practises and an increase in the hatching success rate on commercial farms.
3. All the research findings and conclusions will be incorporated into the current Standards and GOPs to improve crocodile husbandry and farm management practices in South Africa.
4. More optimal farming practises will aid in maintaining a renewable resource on commercial farms which in turn would assist in the conservation of wild Nile crocodiles.
5. Future investigations focusing on the possible causes of embryonic mortality in wild Nile crocodile eggs would benefit significantly from the findings of this study.
6. Scientific publications:
    a. The microstructure of the eggshell and eggshell membranes of the Nile crocodile (*Crocodylus niloticus*).
    b. Changes of the eggshell and eggshell membrane during embryo development in the Nile crocodile (*Crocodylus niloticus*).
    c. Isolation and identification of bacterial and fungal agents associated with commercially farmed Nile crocodile (*Crocodylus niloticus*) eggs and the changes in the microbiome diversity during the incubation period.
    d. Penetration and infection by microorganisms of commercially farmed Nile crocodiles (*Crocodylus niloticus*) eggs.