Component mass as well as calcium and phosphorus content of unbanded and fertile Nile crocodile (*Crocodylus niloticus*) eggs

Geoffrey James Brown

Submitted in fulfilment of the requirements of the degree Philosophiae Doctor (PhD)

Department of Production Animal Studies

Faculty of Veterinary Science

University of Pretoria

20 January 2020

Supervisor: Prof J.O. Nöthling (Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria)

Co-supervisor: Prof P.B.C. Forbes (Department of Chemistry, Faculty of Natural and Agricultural Science, University of Pretoria)

Co-supervisor: Prof J.G. Myburgh (Exotic Leather Research Centre, Faculty of Veterinary Science, University of Pretoria)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

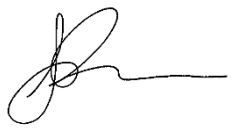
Declaration of originality

I understand what plagiarism is, and I am aware of the University of Pretoria's policy in this regard.

I declare that this thesis is my own original work. Where other people's work has been used (either from a printed source, the Internet, or another source), this has been properly acknowledged and referenced in accordance with departmental requirements.

I have not used work previously produced by another student or any other person to hand in as my own.

I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.



Full name: Geoffrey James Brown

Topic of work: Component mass as well as calcium and phosphorus content of unbanded and fertile Nile crocodile (*Crocodylus niloticus*) eggs

Dedication

For the crocodiles.

> “No mortal creature of all which we know, grows from so small a beginning to such greatness.”

Herodotus, c.484–c.425 BC

Acknowledgements

Mr Stefan van As, owner of Le Croc crocodile farm, for allowing me to collect unbanded egg samples on his farm, and for donating me the valuable fertile eggs used in this research.

Ms. Karon Griffiths, hatchery manager at Le Croc, for her patience while I disrupted her work routine.

Mr Kgomotso Sekwele, laboratory technologist, for his dedicated assistance with sample processing.

My wife Megan, for her encouragement, love and support through this long process.

My co-supervisor, Prof Patricia Forbes, for her guidance, and for always going out of her way to help me with the unfamiliar processes involved in chemical analyses.

And most of all, my supervisor, Prof Johan Nöthling, who showed me by example what it means to be a scientist.

Summary

The Nile crocodile (*Crocodylus niloticus*) is a predator, scavenger and an economically valuable aquaculture species in sub-Saharan Africa. Nile crocodiles are farmed principally for their skins, which are used to manufacture high-value leather goods. By-products of this process, such as meat and fat, also have economic value.

Despite its economic importance, little is known of the Nile crocodile's reproductive physiology beyond behavioural and anatomical descriptions, and some cursory seasonal endocrine profiles. In this manner, *C. niloticus* is not unique: such a paucity of knowledge of the reproductive processes exists for all crocodilian species worldwide, though some species are better researched than others. Furthermore, very little is known about factors that affect egg fertility, foetal survival and resulting hatchling survival. If environmental influences during incubation can be controlled for, the effect of maternally- and paternally-associated factors on embryo, foetal or hatchling survival or performance can be investigated. The egg phase is a critical, self-contained period of the crocodile life cycle, and, compared to the juvenile or adult phases, is an accessible, relatively inexpensive specimen type that can be used to investigate these factors.

During the first half of embryonic development, the formation of the chorioallantois and its fusion with the overlying shell membrane results in a macroscopically visible, circumferential opaque band immediately beneath the shell around the lesser diameter of the egg. This band grows as incubation progresses. Crocodilian eggs that were not fertilised, or which contain embryos that died early in development, have no such visible band, and are referred to as 'unbanded'. In research described in this thesis, the grouping effect of clutch on the mass of the various components (shell, shell membrane, yolk and albumen) of unbanded eggs was evaluated. The effect of potential confounding variables on egg mass and the mass of individual

egg components was assessed. Clutch was found to have a strong grouping effect on egg mass, as well as on the masses of individual unbanded egg components. The mass of each component of unbanded eggs was strongly positively correlated with the mass of the egg. Fertile eggs had substantially lighter yolks and shells than unbanded eggs of similar mass. Controlling for egg mass and incubation period, foetal mass was inversely associated with the mass of the intra-abdominal yolk. The period within the laying season during which an egg was laid, had no significant effect on its mass, nor on the mass of any of its components.

The occurrence of runt hatchlings is a pervasive issue in captive crocodile hatcheries. Such hatchlings are either severely underweight at hatching, or fail to thrive after hatching to the point that they inevitably succumb. Disorders of calcium (Ca) and phosphorus (P) metabolism have been well described in reptiles, and may arise either from insufficient provision, or aberrant metabolism of these elements. Prior researchers found an association between runt *Crocodylus porosus* hatchlings and low plasma Ca (although based on their findings, this relationship was not manifestly causal). Given the potential role of these elements in foetal and hatchling pathologies, the present research sought to measure and describe Ca and P concentration and content (and variabilities thereof) in the various components of unbanded and fertile *C. niloticus* eggs. The grouping effect of clutch on the concentration and content of these elements was determined, and the effect of potential confounders was assessed.

The Ca and P content of the unbanded egg's shell and yolk were influenced principally by the mass of the respective component, and to a lesser extent by the concentration of the element in that component. Contrastingly, shell membrane and albumen Ca and P content were influenced primarily by the concentration of the element. Shell Ca concentration was similar to that of pure calcium carbonate.

Fertile egg yolk Ca content was significantly lower than that of size-matched unbanded eggs, suggesting a net depletion of Ca, however yolk Ca concentration of fertile eggs was found to

exceed that of unbanded eggs in some cases, which could suggest temporary storage of shell-derived Ca. Yolk P concentration and content of fertile eggs was found to be consistently lower than that of unbanded eggs. It was concluded that the yolk is the primary source of P for the developing foetus, while Ca is derived principally from shell and yolk.

Research reflected in this thesis will hopefully be of value in preparation for future research, and in the planning of clinical diagnostic and therapeutic interventions on crocodile farms.

Table of contents

List of figures

List of tables

List of abbreviations

AAS – atomic absorption spectroscopy

AEC – Animal Ethics Committee

ATP – adenosine triphosphate

CAM – chorioallantoic membrane

CAP – chorioallantoic placenta

CFAZ – Crocodile Farmers' Association of Zimbabwe

CI – confidence interval

CITES – Convention on International Trade in Endangered Species

CS – clarity score

DDE – dichlorodiphenyldichloroethylene

DDT – dichlorodiphenyltrichloroethane

ICP-OES – inductively coupled plasma optical emission spectroscopy

IQR – interquartile range

IUCN-CSG – International Union for the Conservation of Nature Crocodile Specialist Group

LOD – limit of detection

LOQ – limit of quantification

NRF – National Research Foundation

PCB – polychlorinated biphenyl

PTH – parathyroid hormone

SD – standard deviation

SE – standard error

TSD – temperature-dependent sex determination

Chapter 1. General introduction and literature review

1.1 Introduction

The Nile crocodile *Crocodylus niloticus* (Laurenti 1768) is an opportunistic apex predator and scavenger in African freshwater ecosystems. It is the largest reptile in Africa, and worldwide is second in size only to *Crocodylus porosus* of Australia and South-East Asia (Alderton and Tanner, 1991). It is one of 26 living crocodilian species (Grigg and Kirshner, 2015), all of which have a tropical or subtropical distribution (Thorbjarnarson, 1996). Crocodilians are classified in the reptilian clade Archosauria, which includes the extinct dinosaurs as well as the extant crocodilians and birds (Thompson *et al.*, 2004). Crocodilians have been divided further by Brochu (2003) into Gavialoidea (gharials) and the Brevirostres (crocodiles, alligators and caiman).

In South Africa and other sub-Saharan African countries, *C. niloticus* is intensively farmed for skin and meat (Fergusson, 2010), however the capacity to produce sufficient quantities of vigorous hatchlings by captive brood stock may represent a potential chokepoint in the profitability and expansion of farming enterprises. In addition to this economic motivation, there is reason to investigate factors affecting crocodile hatchling production from a purely scientific standpoint. To quote Dzialowski and Sotherland (2004):

> "Acquiring in-depth knowledge of the sensitivity of developmental trajectories to environmental perturbations including maternal investment of nutrients and energy in eggs, will improve our understanding of the genesis and importance of maternal effects manifested in phenotypes of hatchlings."

Variations in egg size and shape and the mass and content of egg components may conceivably affect the health and viability of the resulting hatchling, however little is known about how

maternal investment in crocodilian eggs relates to the fitness of the hatchling (Nelson *et al.*, 2010).

Returning to an economic motive, it can be surmised that improvement in the productivity of layer females on a commercial farm may be achieved by (a) increasing the number and proportion of healthy, fertile eggs laid, (b) improving the incubation and hatching processes to decrease the incidence of embryonic death and optimise foetal development, and (c) optimising management of neonates in their transition to independent life, to minimise the occurrence of poor growth or mortality.

Focusing on point (a), before commencing with studies on the effect of treatments or interventions aimed at improving the production of healthy, fertile eggs, it is imperative to accurately describe any natural variation in measures such as egg size, egg mass, and the wet and dry masses of egg components. The potential grouping effect of clutch, and the possible effects of confounding variables must also be described. As an example, a trial may be planned which intends to evaluate the effect of a feed additive for breeding females on yolk size at laying, since maternal diet could conceivably affect egg quality (Webb and Manolis, 1991). Sample selection presents researchers with some important questions. Should a single egg be sampled from a large number of clutches, or should multiple eggs be sampled from a smaller number of clutches? Prior research has shown that clutch effects represent important sources of variation (Webb and Manolis, 1991). Would it be a valid study design, to use females from one breeding pond as a test group, and females from another breeding pond as a control group? Is it necessary to collect all sample eggs at the same time, or can samples be collected piecewise throughout the laying season? Only once the effect of such potential confounding and grouping variables on egg size, component size and other outcome variables are known, can further research be properly planned and samples optimally selected.

Unbanded eggs are those eggs that were never fertilised, or those where the embryo died prior to formation of a visible opaque allantochorionic band immediately beneath the shell. Since they are a by-product of captive hatchling production with no economic value, they are readily available and provide a potentially useful insight into the health of the female and her environment, which in captivity may encompass aspects of animal husbandry.

Chapter 2 of this thesis describes methods developed to open and process both unbanded and fertile eggs. These methods were used to generate specimens used in the research projects described in Chapters 3, 4 and 5.

The first research project, described in Chapter 3 of this thesis, aims to describe the variability in egg mass and the masses of individual egg components of unbanded and fertile eggs, and to assess the grouping effect of clutch and the effects of potential confounding variables on these measurements.

Calcium (Ca) and phosphorus (P) are biological macronutrients essential for life (Lawrence, 2008). Ca plays an essential role in bone and tooth formation, nerve conduction, intracellular transduction of intercellular chemical messages, blood coagulation and as a cofactor in enzymes. P is essential for bone formation, cell membrane phosphoprotein and phospholipid structure as well as the formation of nucleic acids (Shaker and Deftos, 2018).

Disorders of Ca and P metabolism are common in captive reptiles (Marcus, 1981) and have been well described in crocodilians (Lane *et al.*, 1984, Huchzermeyer, 2003, Manolis and Webb, 2016). It is conceivable that an inadequate supply of Ca or P to the developing embryo from an egg that is improperly provisioned with these elements may result in embryonic or foetal death or the birth of a diseased hatchling. It was with this potential pathogenesis in mind, that the second and third research projects forming this thesis were conceived (Chapter 4 and Chapter 5).

The overarching aim of research described in Chapter 4 and Chapter 5 was to describe the concentration and content of Ca and P in unbanded and fertile components of the Nile crocodile egg, characterise the grouping effect of clutch on these measurements, and to investigate the effect of potential confounding variables on these elements. Furthermore, an attempt was made to model the net movement of Ca and P from the freshly laid egg, to the developed foetus by comparing size-matched unbanded and fertile eggs close to the date of hatching.

1.2 The Nile crocodile: habits and ecology

From an evolutionary standpoint, crocodiles and birds are relatively closely related (Grigg and Kirshner, 2015). This relationship can be cautiously exploited by crocodile researchers who, due the current paucity of crocodile-specific research, may be forced to rely on avian research to draw comparisons and conclusions. The proximity of birds and crocodilians is most evident in the reproductive biology of the megapode birds of Australia and New Guinea, which, like crocodilians, produce superprecocial young and do not brood after laying, instead relying on exothermic decay of vegetable matter within a nesting mound to produce the heat required for incubation. At least one megapode species has been reported to have temperature-dependent sex determination as is seen in crocodilians (Göth and Booth, 2005).

Comprehensive accounts of the habits of wild Nile crocodiles have been provided by Cott (1961), Pooley and Gans (1976) and Pooley (1982). Nocturnally aquatic, they come ashore during the day to bask, but may return to water to avoid the midday heat (Cott, 1961). Most hunting takes place from an aquatic environment (Cott, 1961). Prey selection is highly opportunistic and depends on species availability and the size of the crocodile. Hatchling crocodiles feed principally on insects and small amphibians. Davenport *et al*. (1990), in a study of juvenile crocodile feeding behaviour, observed *C. porosus* to explosively jump out of water to catch terrestrial insects. Subadult crocodiles eat fish, water birds, small to medium sized

mammals and reptiles. Fully grown adult crocodiles may feed on large ungulates and even hippopotamus. All size classes eat carrion if available (Pooley, 1982).

The mortality rate of young crocodiles in the wild is high, but a precise figure is difficult to determine since it is likely influenced by environmental and ecological conditions, and tracking of wild crocodiles from hatching to maturity is exceptionally difficult (Pooley, 1982). A suggestion of the maximum allowable mortality was provided by Blake and Loveridge (1975), who estimated that if eggs are collected from wild nests immediately after laying, returning 5% of the resulting young crocodiles at 1 m in length would be adequate to ensure survival of the wild population.

1.3 The Nile crocodile in captivity

In Southern Africa, besides existing as protected populations in national parks and private game reserves, the Nile crocodile is commercially farmed for its skin and meat (Carruthers, 2008). Huchzermeyer (2003) distinguished four categories of crocodilians in permanent or temporary captivity. “Captive” crocodiles are those kept for display, education or research, but which have no productive goal. “Wild-caught” crocodiles are wild crocodiles that are temporarily restrained for research, or prior to slaughter. “Ranched” crocodiles are grown for production of skin or meat but are hatched from eggs collected in the wild. “Farmed” crocodiles are hatched from eggs laid by captive breeding stock.

Fergusson (2010) provides an enlightening review of the conservation status of wild *C. niloticus* populations throughout Africa, together with a summary of recent commercial outputs. Caldwell (2017) provides a more recent summary of trade and export figures, and Isberg *et al.* (2019) has provided further information on the conservation status of wild populations.

In the former Rhodesia and modern-day Zimbabwe, which was at the forefront of Nile crocodile commercial production and research prior to political upheavals (Jenkins *et al.*, 2004), wild-harvested eggs were, and remain, the main source of hatchlings. Rearing stations are concentrated near Lake Kariba, the Zambesi River and its tributaries (Blake and Loveridge, 1975). According to a report compiled for the 2004 IUCN-CSG, in 2003 Zimbabwe exported a total of 115 825 dead or living crocodiles (Jenkins *et al.*, 2004). In 2016, the Crocodile Farmers' Association of Zimbabwe (CFAZ) reported exporting 113 491 skins (Caldwell, 2017). In Botswana, a small number of farms, restricted to the northern region of the country, produce crocodile products from a combination of wild-harvested and captive-laid eggs (Dzoma *et al.*, 2008), and in 2015, importing countries reported obtaining 4400 skins from Botswana (Caldwell, 2017). 42 455 skins were reported by importing countries to have been obtained from South African sources in 2016 (Caldwell, 2017). Importing countries reported receiving 11 876 skins from Mozambique in 2016. South Africa itself imports hatchlings from Mozambique, which likely feature in South Africa's subsequent export figures (Caldwell, 2017).

It is evident from these figures that the Nile crocodile can make a meaningful contribution to the agricultural economies of Southern African countries. Research directed into improving captive breeding productivity may improve profitability of farming enterprises. Furthermore, a better understanding of reproductive physiology obtained by on-farm research could have application in the conservation of wild populations.

1.4 Reproduction in crocodilians

Knowledge of crocodile reproduction is limited, compared to what is known of reproduction in more traditional domestic species. For *C. niloticus* the publications of Cott (1961) and Pooley (1962) are still heavily relied upon for detailed descriptions of reproductive behaviour,

however these are lacking in their description of the underlying reproductive physiology. Khosa *et al.* (2012) described the production of hatchlings from captive-laid eggs as "successful". A search of the available literature revealed very little in the way of scientific research into Nile crocodile hatchling production. This lack of data necessitates extrapolation from research into other crocodilian species, non-crocodilian reptiles and birds.

The most complete summary of research on crocodilian reproduction remains the review by Ferguson (1985) which focused on *Alligator mississippiensis*, although substantial research has been performed since its publication. The review by Thorbjarnarson (1996), which focuses on comparative interspecific aspects of the relationship between female size, egg size, clutch size and reproductive frequency as predictors of reproductive success presents a useful summary from a zoologist's perspective.

1.4.1 The reproductive cycle

Crocodilians have a long reproductive lifespan with a single breeding season per year during which a variable proportion of female animals may breed (Ferguson, 1985). So-called "double-clutching" has been reported in some species (Platt *et al.*, 2012) whereby a single female lays more than one clutch of eggs in a single year. The female Nile crocodile reaches sexual maturity at approximately 1.6–2.3 m in length, while the male is mature at 2.1–3.3 m: this substantial variation was reported from different researchers at different locations (Cott, 1961, Ferguson, 1985, Graham, 1968). Reportedly, female crocodilians experience some reproductive senescence late in life, associated with a reduction in clutch size (Thorbjarnarson and Hernández, 1993). Obese female crocodiles may produce clutches containing fewer eggs (Manolis and Webb, 2016), which was hypothesised to be due either to reduced abdominal space required for a large clutch, or to the endocrine effects of obesity on ovulation rate.

Joanen and McNease (1975) investigated the reproductive biology of *A. mississippiensis,* noting that during the Northern Hemisphere winter months, complete reproductive dormancy occurred. With the onset of spring (March), courtship behaviour occurred, which had a duration of up to three months. In the last month of the spring season, male alligators started producing sperm, and copulation occurred. Oviposition occurred in early summer and hatching occurred in late summer.

In tropical South-East Asia, captive *Crocodylus siamensis* were shown to lay eggs midway through the dry season (February), and continue into the early part of the wet season (June) (Platt *et al.*, 2012). In the Kruger National Park, *C. niloticus* nesting begins in late October and continues to January (Swanepoel *et al.*, 2000).

Multiple paternity has been reported in a variety of crocodilian species, including *A. mississippiensis* (Davis *et al.*, 2001, Lance *et al.*, 2009a), *Alligator sinensis* (Wu and Hu, 2010), *C. porosus* (Lewis *et al.*, 2013), *Caiman latirostris* (Amavet *et al.*, 2008), *Melanosuchus niger* (Da Silveira *et al.*, 2011) and recently also *C. niloticus* (Nöthling, 2019 (personal communication)).

1.4.2 Reproductive anatomy and physiology

Female crocodilians have two ovaries, situated cranioventral to the kidneys and attached to the dorsal body wall (Huchzermeyer, 2003). In *A. mississippiensis* the right ovary is larger than the left and contains more follicles (Lance, 1989).

The reproductive tract of *A. mississippiensis* is divided into seven distinct regions along its length: the anterior infundibulum, posterior infundibulum, uterine tube, uterotubal junction, anterior uterus, posterior uterus and vagina (Palmer and Guillette, 1992). These anatomical regions are similar to those found in birds, but quite different to those of non-archosaur reptiles. After ovulation, the yolk travels through the infundibulum. The albumen is formed in the

uterine tube. The shell membranes are formed in the anterior uterus, and mineralisation of the shell occurs in the posterior uterus. However, unlike birds, which lay their eggs sequentially as they are formed, female crocodilians retain the entire clutch of eggs within the tubular genitalia until they are simultaneously oviposited (Palmer and Guillette, 1992).

Follicular growth in reptiles involves multiple histological and physiological changes, including proliferation of yolk, development of the oocyte as well as changes in the granulosa and thecal cells (Uribe and Guillette, 2000). In *A. mississippiensis*, the pre-ovulatory oocyte was reported to range from 5 mm to 45 mm in diameter (Lance, 1989), however in this study no indication was provided of the mean size or range immediately prior to ovulation. Using ultrasonography, a direct correlation was found between the size of *A. mississippiensis* follicles and serum testosterone and oestradiol concentration (Lance *et al.*, 2009b). The size of corpora lutea forming subsequent to ovulation can indicate recent reproductive activity (Guillette *et al.*, 1995): those with a diameter of at least 4 mm likely occur subsequent to an ovulation in the year of examination.

Serum oestradiol in alligators has been shown to increase from basal levels (< 367 pmol/L) to around 1836 pmol/L in April (Northern Hemisphere spring), reaching a peak in May. A similar pattern of testosterone production was shown in males, where a peak serum testosterone concentration of just under 5.20 nmol/L was reached in May. Serum progesterone was shown to increase in alligators around the time of ovulation, from basal (< 7.95 nmol/L) to a maximum of 50.88 nmol/L in early June, which corresponded with the period around egg-laying (Lance, 1989). These steroid hormone profiles reflect earlier behavioural observations of Joanen and McNease (1975) and other researchers.

In research conducted on Nile crocodiles in Zimbabwe, males showed an increase in serum testosterone in June, July and August (from < 1.7 nmol/L to between 6.9 and 27.7 nmol/L), while in the female, oestradiol showed an increase during the same period from basal

(< 367 pmol/L) up to a level as high as 3671 pmol/L. Progesterone followed a rather scattered distribution throughout the year, however levels were uniformly low during the autumn period (March–May), with samples reaching peaks of up to 8.26 nmol/L through the remainder of the year (Kofron, 1990). A marked difference in peak plasma progesterone levels between *A. mississippiensis* and *C. niloticus* is evident: Lance (1989) speculated that Kofron (1990) may have missed a transient rise in plasma progesterone with monthly sampling of *C. niloticus* females.

1.4.3 Nesting and commercial egg collection

Depending on species, crocodilian eggs are deposited in holes dug by the female (as in *C. niloticus, Crocodylus johnstoni, Crocodylus intermedius* and *Gavialis gangeticus*), or in nesting mounds constructed prior to laying, (as in *C. porosus, A. mississippiensis, A. sinensis, Tomistoma* and *Caiman* spp.) (reviewed by Ferguson (1985) and Ackerman and Lott (2004)).

Hatchlings for commercial growing may be produced from eggs derived from wild nests, or from eggs laid by captive females (Thorbjarnarson, 1999). In South Africa, farms that practice captive breeding typically house adult crocodiles in communal ponds, around which are artificial nesting banks of soft sand. Taking care to keep adult crocodiles at bay, eggs are dug up from nests by farm staff and laid in their original orientation in an incubation box as described by Manolis and Webb (2016), surrounded by a substrate such as vermiculite. These eggs are then transported to a temperature and humidity-controlled incubator. Researchers have recommended that transport should be avoided between eight and 16 days after laying or close to the time of hatching, since vibration or trauma during these periods may cause detachment of the allantochorion and foetal death in early incubation, or premature hatching in late incubation (Bock *et al.*, 2004). Trauma-induced early embryonic death occurs due to the shearing of delicate blood vessels joining the chorion with the outer layer of the allantois (Ferguson, 1982, Joanen and McNease, 1987, Chabreck, 1978, Blake and Loveridge, 1975).

1.4.4 Incubation temperature, hatchling phenotype, health and growth rate

Vertebrates that do not have distinct differences in sex chromosomes may exhibit temperature-dependent determination of phenotypic sex (TSD) (Bull and Vogt, 1979). The occurrence of TSD in extant crocodilian species has been reviewed by Lang and Andrews (1994).

Ackerman and Lott (2004) summarized factors contributing to the within-nest variation in temperature through the incubation period in reptiles. The temperature of the nest is influenced by metabolic heat produced by the developing embryo or foetus (which in reptiles is negligible until late incubation), and soil temperature. Soil temperature in natural nests is in turn affected by the position of the nest (shaded or exposed), its depth, soil heat conduction characteristics, soil moisture, season, and diurnal sinusoidal variation. In crocodile egg hatcheries, much of this source of variability is removed when eggs are kept under controlled conditions, usually buried in a substrate such as vermiculite and kept at a constant temperature and humidity.

In *C. niloticus*, Hutton (1987) found that, if temperature-controlled laboratory incubation was commenced at between day 0 and day 30 of incubation, a temperature of 28 °C resulted in only female offspring. Eggs incubated at a mean temperature of 31 °C also resulted in only female offspring. Eggs incubated at 34 °C resulted in a preponderance of male offspring, and a small minority of female offspring.

In addition to his findings on temperature-associated sex differentiation, Hutton (1987) also found that Nile crocodile eggs incubated at higher temperatures (34 °C) had incubation periods that were substantially (around 25 days) shorter than those incubated at lower temperatures (28 °C). Hatching success was highest at 31 °C, and lowest at 34 °C. Eggs incubated at higher temperatures produced hatchlings with shorter total length that grew faster than their longer, lower-temperature siblings. The researcher speculated that this difference in post-hatching growth rate could be because hatchlings incubated at higher temperatures utilised less of their

intra-abdominal yolk during incubation, which was then available to sustain them in the post-hatching period (Hutton, 1987).

Webb and Smith (1984), cited in Webb and Cooper-Preston (1989), found that incubation temperature affected the survival rate of *C. johnstoni*. Webb *et al.* (1983b) found that *C. johnstoni* hatchlings from eggs incubated at 34 °C exhibited a far higher rate of anatomic abnormalities than those from eggs incubated at 30 °C. At lower temperatures (26–28 °C), all embryos died prior to hatching, with a high incidence of jaw and tail abnormalities as well as runting. These researchers theorised that there is an optimal average temperature of around 31–32 °C at which incubation should take place to avoid such defects. This "optimal temperature theory" was confirmed by research done in *A. mississippiensis* by Joanen *et al.* (1987). In *A. mississippiensis, Crocodylus palustris, C. johnstoni, C. porosus* and *Caiman crocodilus*, an incubation temperature of 31 °C can be expected to produce more females, and a temperature of 32 °C can be expected to produce more males (Lang and Andrews, 1994).

Whitehead *et al.* (1990) also investigated the effect of incubation temperature on embryo development of *C. johnstoni*. They found that a sigmoid growth curve existed at all incubation temperatures from 28 °C to 33 °C, that growth rates were substantially faster at higher temperatures, and that hatchlings from eggs incubated at higher temperatures tended to be smaller with a greater quantity of residual yolk. The period of incubation that was most sensitive to temperature-associated effects was the period of organ differentiation during early incubation. Organ development occurred much faster at higher temperatures, but was not accompanied by a temperature-associated increase in absolute mass. Once this differentiation phase was complete, the growth rate appeared to be far less dependent on temperature.

Piña *et al.* (2007) showed that *C. latirostris* hatchlings incubated at 31 °C were longer and hatched later than those incubated at 29 and 33 °C, but that body mass was similar among incubation temperatures. This suggested that earlier hatching is accompanied by a greater

provision of intra-abdominal yolk. Neither initial body mass nor clutch affected post-hatching growth, however hatchlings incubated at 33 °C grew more slowly during the first year than those incubated at 29 or 31 °C.

Allsteadt and Lang (1995) found that *A. mississippiensis* hatchlings incubated at 32 °C were larger than those incubated at 29 °C. In contrast to the studies of Hutton (1987) and Piña *et al.* (2007), in this study the relationship between residual yolk mass and incubation temperature was not immediately clear: larger yolk masses occurred at 31 and 33 °C, whereas smaller yolk masses occurred at 29 and 32 °C. The residual yolk of female hatchlings exceeded that of males, but males were on average larger. Importantly, clutch had a strong influencing effect on all egg and hatchling variables, suggesting that maternal factors play an important role in determining hatchling phenotype.

As well as affecting post-hatching growth rate, incubation temperature was also shown to affect skin pigmentation: skin pigments were deposited earlier in incubation in those eggs subjected to higher temperatures (Deeming and Ferguson, 1989, Murray *et al.*, 1990).

1.4.5 Hydration state and partial pressures of gases

Temperature is probably not the only nest-associated factor with influence on embryo health and survival: water loss and gain and gas tension (oxygen and carbon dioxide) likely also have significant effect (Whitehead, 1987). Lutz *et al.* (1980) studied the relationship between the hydration state of unbanded *Crocodylus acutus* eggs and their gas permeability. As the water content of shell and membrane increased, there was a corresponding marked decrease in gas permeability. Crocodilian eggs have a much greater water vapour conductance than birds' eggs (according to Packard *et al.* (1979), eggs of *A. mississippiensis* conduct water at a rate five times that of avian eggs of similar size), but are able to replenish lost water using ambient water vapour if placed in a humid environment (Grigg, 1987).

In *C. johnstoni* and *C. porosus*, maximum consumption of oxygen does not occur in the foetus immediately prior to hatching, but peaks when incubation is less than 90 percent complete (Whitehead *et al.*, 1990). In *C. niloticus* eggs incubated at 31 °C, oxygen consumption increases in a roughly linear fashion from day 53 to day 80 of incubation, plateaus from day 80 to approximately day 95, whereupon it declines to hatching at day 100 (Aulie and Kanui, 1995). Alligator foetuses were shown to stop growing during the last week of incubation prior to hatching (Nelson *et al.*, 2010, Ferguson, 1985), entering a phase of dormancy during which they internalised the yolk, closed the yolk scar and possibly gathered strength for the process of egg emergence.

Flexibility in the rate of development, sex ratio and timing of hatching may afford crocodiles the capacity for short-term adaptations, which could improve population survival rates under adverse circumstances (Thompson, 1989, Shine and Brown, 2007). In particular, the synchronisation of hatching may allow the female to assist hatchlings to emerge from the nest (Thompson, 1989).

1.5 Crocodilian eggs

The amniote egg is a self-contained package that must nourish the developing embryo and provide it with all its growth and maintenance requirements until the hatchling is able to feed independently. Additionally, it must protect the embryo from predation by insects, dehydration, and microbial invasion (Thompson *et al.*, 2004).

The composition of the eggs of oviparous species varies. It has long been recognised that avian species that produce precocial young lay eggs with larger yolks, higher energy density and less water than those species that produce altricial young (Carey *et al.*, 1980, Tarchanoff, 1884).

C. niloticus eggs are tough and ellipsoidal, with a hard, calcareous shell, a thick shell membrane, and in infertile or recently laid fertile eggs, a jelly-like transparent albumen. The

yolk is surrounded by a very thin, easily ruptured vitelline membrane (Pooley, 1982). This general description seems to be applicable to other crocodilian species: a similar account was given for *C. porosus* by Burley *et al.* (1988).

Crocodilian eggs are laid at a relatively late stage of development. In *A. mississippiensis* an embryo within a newly laid egg consists of between 9 and 20 somites, a notochord, an obvious primitive streak and undifferentiated brain and otic vesicles. It lies on top of the yolk and is not attached to the overlying shell membrane (Ferguson, 1985). Head measurements have been used as a method of staging *A. mississippiensis* foetuses (Deeming and Ferguson, 1990). According to Peterka *et al.* (2010), in *C. niloticus* the degree of maturation as measured by head morphometrics correlates more closely with body mass than it does with incubation period.

Length of the incubation period varies with environmental temperature. *C. niloticus* eggs incubated at 28 °C took approximately 111 days to hatch, while those incubated at 34 °C took approximately 84 days to hatch (Hutton, 1987). *C. johnstoni* eggs incubated at 29 °C took 100.9 days (SD 1.8 days) to hatch, while those incubated at 33 °C took 68.4 days (SD 1.5 days) to hatch (Whitehead *et al.*, 1990).

Early studies of unbanded and fertile *C. niloticus* eggs and hatchlings were broadly descriptive (Pooley, 1969) and confined to wild populations (Bigalke, 1931, Modha, 1967). Little further published research has been done on the eggs of this species with the exception of Hutton (1987) who investigated temperature-dependent sex determination.

1.5.1 Egg size and hatchling size

Little specific research has been done on the size and mass of crocodilian eggs and their components. Rather, such measurements are taken as part of research into other aspects of egg physiology, and must be extracted from these reports.

Alligator mississippiensis eggs were measured at three locations in the southern United States. A mean egg length of 7.25 to 7.57 cm, with a mean width of 4.08 of 4.47 cm was reported (Cardeilhac *et al.* (1999), cited by Huchzermeyer (2003)). In a study of the effect of incubation temperature on *A. mississippiensis* hatchlings, Allsteadt and Lang (1995) weighed eggs from three clutches. Mean egg mass per clutch varied from 74.65 g (SD = 3.53 g, n = 51) to 78.0 g (SD = 2.40, n = 41). Garnett and Murray (1986), in a study of factors affecting the growth of captive *C. porosus* hatchlings, used eight clutches of eggs with clutch mean masses ranging from 65.5 g (SD 1.2 g) to 117.2 g (SD 4.2 g). In a study of the chemical composition of *Crocodylus novaeguineae* eggs, Jenkins (1975) analysed samples from eggs that ranged in mass from 45.7 g to 84.6 g. Nöthling *et al.* (2019b) used a computer model to estimate the volume of 316 *C. niloticus* eggs from shells after hatching. They found a median egg volume of 99.9 ml (upper boundary of lower quartile: 92.6 ml, lower boundary of upper quartile: 106.1 ml).

It is evident from the data presented in the previous paragraph that, of the crocodilian species studied so far, eggs vary considerably in size (and mass) within species. The same is true for bird species. Most variation in bird's egg mass has been found to occur between, rather than within clutches. It has been found that female birds tend to produce successive clutches containing eggs of similar size. Older laying hens were shown to produce heavier eggs. In chickens, ducks and turkeys, it has been shown that intermediate-sized eggs have the best hatching rate (Christians, 2002).

In general, it seems as though bigger crocodilians produce bigger eggs, both among and within species. Greer (1975) found within a limited sample set that species attaining a greater total length, tended to produce larger clutches with bigger eggs, which in turn produced larger hatchlings. For *A. mississippiensis*, Deitz and Hines (1980) found that mean egg mass per clutch was positively associated with clutch size (but the relationship was loose: clutch size

explained just 49% of mean egg mass). Thorbjarnarson and Hernández (1993) found that larger female Orinoco crocodiles laid greater numbers of larger eggs earlier in the season than smaller females, and were less likely than smaller females to be reproductively quiescent in a given season. The relationship between female size, clutch size and egg size was different in birds and crocodiles to the finding in oviparous snakes by Ford (1989), who noted that if female size was kept constant, there was an inverse relationship between clutch size and egg size. *C. johnstoni* eggs were reported to contain a greater proportion of yolk and a smaller proportion of albumen than *C. porosus* and *A. mississippiensis* (Manolis *et al.*, 1987), however these findings should be interpreted with caution, since these authors compiled their figures from the research studies of others. Furthermore, sample numbers were low and the potential effects of confounding variables were not taken into account. Regardless, it seems likely that varying evolutionary pressures on different crocodilian species have resulted in differences in the composition of their eggs.

In *A. mississippiensis* and *C. porosus,* it was found that eggs tended to have greater variation in mass between clutches than within clutches (Deitz and Hines, 1980, Garnett and Murray, 1986), however for *C. latirostris*, Stoker *et al.* (2013) found that the within-clutch variation in egg mass was greater than the mean egg mass variation among clutches, and Pooley (1962) reported that there can be significant variation in egg size within clutches.

Deitz and Hines (1980) found that the mean egg mass within a clutch of *A. mississippiensis* eggs was positively related to the mean mass of hatchlings within that clutch, a finding consistent with the later findings of Garnett and Murray (1986) for *C. porosus*. Furthermore, the mean hatchling mass of *A. mississippiensis* clutches varied more than the mass of individual eggs within clutches, a finding that was again consistent with findings by Garnett and Murray (1986) for *C. porosus*. The latter researchers found that the mean hatchling mass of eight

clutches varied by clutch from 40–80 grams, and that even within a clutch, there was a substantial variation in hatchling mass (SD varying from 16–20 g).

In *C. johnstoni,* Webb *et al.* (1983b) showed that for each increase by one gram in egg mass, hatchling mass is expected to increase by 0.62 g, and that egg mass explains 89% of variation in hatchling mass. For *C. porosus*, Webb *et al.* (1983a) demonstrated that hatchlings increased by 0.64 g for each additional gram in egg mass, and that 79% of the variation in hatchling mass was explained by egg mass. These findings were very similar to those of Deeming and Ferguson (1989), who found that the masses of *A. mississippiensis* hatchlings in their final stage of incubation correlated strongly with the initial mass of the egg ($R^2 = 0.78$, coefficient of initial egg mass 0.635), and that hatchling mass constituted 66.76% (SD 3.55%) of the mass of the egg. Isberg *et al.* (2005) found that egg length had a significant effect on hatchling head length, snout-vent length, total length and belly width of *C. porosus*.

In an investigation of factors affecting the survival of *C. porosus* hatchlings in captivity, Brien *et al.* (2014) found that hatchling body mass at four days of age is expected to increase by 0.54 g for each one gram increase in egg mass. ($F = 48.74$, $R^2 = 0.83$, $P < 0.0001$).

The mass of individual emu (*Dromaius novaehollandiae*) egg components increased significantly as egg mass increased, however the relative contribution of albumen and yolk to eggs did not vary with egg mass. Hatchling mass increased significantly with egg mass, and, independent of initial egg size, larger hatchlings had less residual yolk than did smaller hatchlings (Dzialowski and Sotherland, 2004). Brien *et al.* (2014) found that lighter *C. porosus* hatchlings compensated to some extent by growing faster than heavier hatchlings: the authors speculated that this could be as a result of consumption of a greater quantity of residual yolk.

Nöthling *et al.* (2019a) developed a method to estimate egg volume from scale photographs of hatched egg remnants, and used it to investigate the relationship between estimated egg volume

and hatchling mass (Nöthling *et al.*, 2019b). They found that hatchlings from the same clutch tend to be very similar in mass. However, if the grouping variable of clutch is not considered, there is considerable variation in hatchling size for a given egg size. Overall, there was a strong positive linear relationship between hatchling mass and the estimated volume of their eggs of origin ($R^2 = 0.85$, n = 316). These researchers used the relationship between estimated egg volume and hatchling mass to introduce a new concept, namely the 'productivity' of the egg. They found that the productivity of *C. niloticus* eggs varied from 0.57 grams of hatchling per ml of egg, to 0.81 g/mL. The productivity was influenced by clutch: 54.6% of variation occurred within clutches and 45.4% of variation occurred among clutches.

1.5.2 Eggshell

Romanoff and Romanoff (1949) and Terepka (1963) provided early descriptions of the microscopic structure of the avian eggshell, but a more recent review of eggshell formation in birds, with some reference to reptiles, has been provided by Hincke *et al.* (2012).

The calcareous eggshell protects the egg content from microbial and mechanical damage as well as desiccation, while allowing the exchange of gases through the pores (Nys *et al.*, 1999). In *A. mississippiensis*, the mass of the shell constitutes approximately ten percent of the wet mass of the egg (Nelson *et al.*, 2010). During eggshell formation in crocodilians, Ca is withdrawn from bone (Wink and Elsey, 1986, Lance *et al.*, 1983) and from dermal scutes (Dacke *et al.*, 2015) and is deposited in the form of calcium carbonate on the outer fibres of the shell membrane from a supersaturated solution in the shell gland (Hincke *et al.*, 2012).

In *C. porosus*, the combined mass of shell and shell membrane was shown to increase by 7.27 g for each increase by 1 cm in breadth of the egg (SE = 0.91 g, $R^2 = 0.73$, n = 30), and by 2.98 g for each 1 cm increase in egg length (SE = 1.14, $R^2 = 0.58$, n = 30). In *C. johnstoni*, the combined mass of shell and shell membrane was shown to increase by 2.97 g for each increase

by 1 cm in egg breadth (SE = 0.68, R^2 = 0.46, n = 44), and by 1.81 g for each increase by 1 cm in egg length (SE = 0.74, R^2 = 0.37, n = 43) (Manolis *et al.*, 1987).

In a recent research paper into the composition of a small sample of unbanded and fertile *C. latirostris* eggs, Leiva *et al.* (2018) found that unbanded eggshell and shell membrane contributed 19.5% (SD 2.6%) to total egg mass.

According to Ferguson (1982), the calcified eggshell varies in structure from the surface to the shell membrane. This researcher examined the shell and shell membranes using electron microscopy and characterised the crystalline structure of the eggshell using energy dispersive X-ray analysis, determining that the alligator eggshell is made up principally of calcite ($CaCO_3$) crystals. At the surface, there is an outer, densely calcified layer consisting of calcite crystals with their long axes oriented at right angles to the shell surface. Immediately beneath this layer exists the honeycomb layer, which has the calcite crystal axes oriented parallel with the surface. Beneath the honeycomb layer is a very thin organic layer, and beneath this layer lie the mammillary knobs. The mammillary knobs are the nucleation sites of initial calcification of the shell on the outer shell membrane (Solomon *et al.*, 1994). Using atomic absorption flame spectroscopy (AAS) and measuring in triplicate from three separate samples derived from single eggs analysed at regular intervals after laying, Ferguson (1982) found that an infertile (unbanded) alligator eggshell contained on average 31.7% Ca, while the opaque banded portions of shells from fertile eggs varied from 33.2 to 41.4% Ca. He found that the difference in Ca concentration between opaque and non-opaque zones was not statistically significant.

Marzola *et al.* (2015) added to Ferguson's work on crocodilian eggshell structure by describing and comparing the appearance of the pores and surface appearance of the eggshells of *A. mississippiensis*, the Philippine crocodile *Crocodylus mindorensis* and the smooth-fronted caiman *Paleosuchus palpebrosus*. Kern and Ferguson (1997) confirmed earlier findings by Ferguson (1982), namely that the equator of the eggshell had far more pores per surface area

than the ‘shoulders’ of the egg, which in turn had far more than the poles. Stoker *et al.* (2013) found that *C. latirostris* eggs with fewer pores per surface area were associated with reduced embryo survival. Wink and Elsey (1994) and Wink *et al.* (1990) compared infertile and fertile eggshells of the Chinese alligator *A. sinensis* and the American alligator *A. mississippiensis,* noting thicker shells with fewer open pores in infertile eggs.

Cedillo-Leal *et al.* (2017) found that *C. latirostris* eggs with rougher shells were more tolerant of being submerged in water early in development: this was thought to be due to the entrapment of air bubbles at the eggshell surface.

The chalky white opaque band that forms around the lesser circumference of the fertile egg in crocodilians has been well described. The band is created by changes to the shell and shell membrane caused by the developing chorion. Initially it starts as a focal spot, but expands to form a ring around the lesser circumference of the egg. It widens to encompass the entire egg by the end of incubation (Ferguson, 1985). This band is used by crocodile farmers to differentiate fertile from infertile eggs. In *C. niloticus* eggs, the development of this band is non-linear over the course of incubation (Nöthling, 2018 (personal communication)).

Embryonic death can be identified in eggs that have unexpected cessation of band growth, or narrower bands than those of their clutchmates. Infected or rotten fertile eggs may exhibit black or green blotching of their bands (Ferguson, 1985).

Crocodilian eggs incubated in very moist environments may develop longitudinal cracks in their shells. Cracked eggs lose water four to five times quicker than uncracked eggs kept in the same conditions (Grigg, 1987).

Ferguson (1981) found that acids produced by bacteria in the mound nests of alligators assisted in the extrinsic dissolution of the calcified shell, which progressively weakens the eggshell as incubation progresses, allowing for more gas and water vapour conductance and ultimately

easier hatching. The withdrawal of solids from the eggshell during foetal development was posited by Whitehead (1987) to alter the conductance of the eggshell to oxygen and carbon dioxide, which would moderate the exposure of the foetus to hypoxia and hypercapnia as incubation progresses.

Kern and Ferguson (1997) incubated fertile eggs in two groups, at 30 and 33 °C respectively. For both of these temperature groups, the thickness of the eggshell decreased through incubation. The reduction in shell thickness was most pronounced at the poles of the egg at both temperatures, however there was significant reduction at the equator and the 'shoulders' of the egg as well. There was a greater thinning effect at eggs incubated at 33 °C. The process of eggshell thinning did not appear to be very noticeable until approximately 40 days of incubation, whereupon it began in earnest.

1.5.3 Shell membrane

The shell membrane of crocodilian eggs is much thicker than that of birds' eggs (Ferguson, 1982, Lutz *et al.*, 1980). In *A. mississippiensis* it has been reported as 150–250 µm (Ferguson, 1982) or 50–216 µm thick (Kern and Ferguson, 1997). That of *C. acutus* was reported by Lutz *et al.* (1980) to be 430 µm thick. The shell membrane of *A. mississippiensis* was reported to be formed of many layers of fibres, each running haphazardly in different directions (Ferguson, 1982). The innermost surface of the shell membrane is covered by a surface coating 3–8 µm thick, referred to by Kern and Ferguson (1997) as the limiting membrane. This membrane abuts against the albumen in the freshly laid egg.

The fibres forming the shell membrane are approximately 0.5–2.0 µm in diameter, and were shown to expand or contract in response to moisture level. Shell membrane is tightly attached to the shell at the beginning of incubation, but is loose and easily detachable near the time of hatching (Kern and Ferguson, 1997).

1.5.4 Yolk

Yolk contains most of the nutrients and chemical energy required by the developing embryo (White, 1991, Thompson *et al.*, 2004). In *A. mississippiensis*, most hatchling solids are derived from the yolk (Nelson *et al.*, 2010). Crocodilian yolk consists principally of proteins and fats which are oxidatively catabolised for energy (Whitehead, 1990). When adjusted for egg-size, the mean dry mass of yolk of *A. mississippiensis* declined with duration of incubation (Packard and Packard, 1989), indicating consumption by the developing foetus.

However, the maternal provisioning to oviparous species by the yolk extends beyond mere nutrition. Schwabl (1993) demonstrated that the yolk of zebra finches (*Taenopygia guttata)* and canaries (*Serinus canaria)* contained testosterone in relatively high concentration which did not appear to influence sex differentiation. In canaries, yolk testosterone concentration had a positive association with the hatchling's social rank.

The maternal liver synthesises protein-lipid complexes which will form the substance of the yolk: these are predominantly vitellogenin and VLDLγ (Thompson *et al.*, 2004).

According to Manolis *et al.* (1987), at 1.058 g/cm^3 for *C. johnstoni*, 1.042 g/cm^3 for *C. porosus* and 1.038 g/cm^3 for *Alligator missippiensis*, the yolk of crocodilian eggs is of greater average density than the figure for yolk density of 1.035 g/cm^3 reported by Romanoff and Romanoff (1949) for the domestic fowl. However, these data were collated from different research studies and were not formally statistically compared.

Nelson *et al.* (2010) showed that yolk mass of *A. mississippiensis* increases with increasing egg mass and that alligator eggs contained a substantially greater proportion of yolk than chicken eggs. Wet alligator yolk mass contributed approximately 47% to total egg mass (mean yolk mass 35.95 g (SD 3.82 g)). For each gram that egg mass increased, wet yolk mass of *A. mississippiensis* eggs increased by a mean value of 0.41 g ($R^2 = 0.83$) (Nelson *et al.*, 2010).

A sample of nine *C. porosus* egg yolks was found to weigh an average of 31 g in a study by Burley *et al.* (1988). Another study by the same authors reported the mass of yolk in seven samples of *C. porosus* to have a mean value of 29.6 g (range: 24.0–49.7 g), which constituted a mean of 36.5% of total egg mass. No indication of variability was provided for mean egg mass (Burley *et al.*, 1987).

In *C. porosus*, the mass of the yolk was calculated to increase by 19.3 g for each increase by 1 cm in egg breadth (SE = 2.25 g, $R^2 = 0.74$, n = 19), and by 9.24 g for each increase by 1 cm in egg length (SE = 3.45 g, $R^2 = 0.61$, n = 22). In *C. johnstoni*, yolk mass was calculated to increase by 19.38 g for each increase by 1 cm in egg breadth (SE = 1.71 g, $R^2 = 0.86$, n = 37), and by 9.38 g for each increase by 1 cm in egg length (SE = 3.58 g, $R^2 = 0.39$, n = 38) (Manolis *et al.*, 1987). Leiva *et al.* (2018) found that *C. latirostris* yolk contributed 52.1% (SD 3.7%) to total egg mass.

Ahn *et al.* (1997) showed that young laying hens (28 weeks of age) produced eggs with the lowest ratio of yolk to albumen, while 55 to 78-week-old hens produced the highest ratio, and that of old hens (97 weeks) was intermediate. Furthermore, they showed that larger chicken eggs contained on average a lower ratio of yolk to albumen than smaller eggs. No such information exists for crocodilian species. Nelson *et al.* (2010) found that the chicken egg yolk comprised 28.8% of total egg mass, with a mean mass of 17.14 g (SD = 2.54 g). The chicken yolk mass increased with increasing egg mass: for each gram that egg mass increased, yolk mass increased by a mean of 0.37 g ($R^2 = 0.52$).

Finkler *et al.* (1998) found that aspirating approximately 20% of the mass of a chicken egg yolk had no effect on hatchling mass, however the researchers postulated that removing yolk from the egg may decrease the nutrition available to the neonate, thereby reducing its survival after hatching.

Whitehead (1990) showed that intra-abdominal yolk was sufficient to sustain the energy needs of resting *C. johnstoni* hatchlings for up to four weeks without obvious deterioration in body condition, while simultaneously allowing for increase in body length.

These findings show that, in general and across species, yolk tends to increase in mass with increasing egg mass. However, for those studies where such information is available, there also appears to be substantial variability in yolk mass for a given egg size. Furthermore, there are few studies on the variability in yolk composition, which could have differential influence on the nourishment of the embryo, foetus or hatchling.

1.5.5 Albumen

In birds' eggs, albumen is the primary source of water for the developing embryo. Its solid component consists principally of specialised albumen proteins in addition to trace elements, carbohydrates and vitamins (Sotherland and Rahn, 1987). Unlike birds' eggs, crocodilian eggs have no chalazae that extend through the albumen to the yolk (Ferguson, 1982).

Albumen has been shown to contribute to avian hatchling biomass, particularly in the latter part of incubation (Muramatsu *et al.*, 1990). Two mechanisms of albumen uptake have been proposed, which are summarized by Nelson *et al.* (2010): One hypothesis (Deeming, 1991) proposes that a hole forms in the amnion, and the albumen mixes with the amniotic fluid, from where it is ingested by the embryo. The second hypothesis (Yoshizaki *et al.*, 2002) proposes that the chorion, yolk sac membrane and amnion actively transports albumen water and solids by endocytosis into the yolk sac and amnion, from where they are either absorbed or ingested by the embryo.

The albumen of hens' eggs was shown by Nelson *et al.* (2010) to contribute the greatest amount to egg mass (a mean contribution of 61.9%, with a mean albumen mass of 36.81 g, SD 3.17 g). Chicken albumen mass was positively associated with egg mass (coefficient 0.554, R^2=0.739).

In the same study, Nelson *et al.* (2010) showed that alligator albumen mass was positively associated with egg mass, with coefficient 0.52, and a very high R^2 value of 0.90. Albumen of *A. mississippiensis* was found to contain approximately 96% water. The mass of albumen increased with increasing egg mass at a rate faster than that of yolk mass (Nelson *et al.*, 2010), which was similar to the findings of Finkler *et al.* (1998) for the domestic fowl.

The mean mass of the albumens in a sample of seven *C. porosus* eggs was 41.6 g (range: 32.7–53.0 g), which constituted a mean value of 51.2% of total egg mass (Burley *et al.*, 1987). For *C. latirostris*, Leiva *et al.* (2018) found that albumen contributed 28.5% (SD 5.1%) to total egg mass.

Albumen of *C. porosus* was described as stickier and firmer than that of the hen's egg (Burley *et al.*, 1987), despite having a lower proportion of solids. The researchers suggested that this was because of the presence of a hydrophobic, potentially antimicrobial protein, which they called 'crocalbumin'.

In another study on the eggs of *C. porosus*, albumen mass was shown to increase by 23.65 g with each increase by 1 cm in egg breadth (SE = 3.20, R^2 = 0.67, n = 21), and by 13.01 g with each increase by 1 cm in egg length (SE = 3.38, R^2 = 0.62, n = 19). In *C. johnstoni*, albumen mass was shown to increase by 13.77 g for each increase by 1 cm in egg breadth (SE = 2.12, R^2 = 0.66, n = 40), and by 8.73 g with each increase by 1 cm in egg length (SE = 2.64, R^2 = 0.50, n = 38) (Manolis *et al.*, 1987).

The albumen water available for development may restrict the growth of the embryo: in a study on the domestic fowl by Finkler *et al.* (1998), reducing the albumen content of eggs by approximately 20% was found to reduce the wet mass of the near-term embryo as well as the length of the tibiotarsus, while reducing yolk mass by the same amount did not alter hatchling mass.

Albumen makes up between 42 and 47% of total egg mass in *C. porosus*, *C. johnstoni* and *A. mississippiensis* (Tracy and Snell, 1985, Ferguson, 1982), cited in Manolis *et al.* (1987). Chicken egg albumen was shown to contain a greater fraction of solids than alligator egg albumen, and albumen contributed approximately 43% wet mass to whole egg mass (Nelson *et al.*, 2010), with a mean mass of 33.13 g (SD 4.54 g).

From the above studies, it appears that the proportional contribution of wet albumen to total egg mass is far more variable than that of wet yolk. Since albumen consists principally of water, and crocodilian eggs are incubated in enclosed, humid nests, it seems likely that water vapour may move to and from albumen during development. The effect of environmental humidity may serve as an important confounder in determining the mass proportion of albumen, which may in turn impact embryonic or foetal development.

1.6 Ca and P in the developing embryo

Ca is essential for normal physiological function in animals. In higher organisms the majority of Ca is contained in bone and teeth together with P in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], and a small quantity can be found in intra- and extracellular fluids, where its concentration is kept within tightly controlled limits by movement to and from bone deposits (Carafoli, 1987). Ca is continuously removed from cells by an energy-dependent transport mechanism: any disease process that disrupts this active process can lead to cell death (Nicotera *et al.*, 1992).

1.6.1 Component Ca and P concentration and content

Archosaur embryos mobilise substantial quantities of Ca from extra-embryonic sources during the second half of incubation (Packard and Packard, 1989). In oviparous species, this must originate from the yolk or must be obtained from the shell, across the chorioallantoic membrane (CAM) (Packard and Clark, 1996). In lizards and snakes, most Ca comes from the yolk. In

crocodilians most Ca comes from the shell, however yolk may serve as a temporary storage for shell-derived Ca so that the residual yolk at hatching contains as much or even more Ca than the yolk of the freshly-laid egg (Packard and Packard, 1989).

In a study of *A. mississippiensis*, Packard and Packard (1989) sampled one egg from each of six clutches on days 0, 16, 33, 40, 49, 57, 65 and 73 after laying, and analysed component Ca, P and Mg using atomic absorption spectroscopy (AAS). Mean foetal Ca content remained consistently low until approximately day 40, when it began increasing rapidly until hatching. Mass-adjusted mean foetal P content noticeably increased from approximately day 15 of incubation, and began to increase rapidly from about day 40. Yolk Ca content did not substantially change from laying until day 33, whereupon it began to decline in a steep linear fashion, but from day 65 until hatching it increased rapidly again. The P content of the yolk was shown to decline linearly from day 33 onwards, however at hatching there was a substantial quantity of P present in residual yolk. In their discussion of results, the researchers noted that it appeared that all embryonic P was obtained from yolk stores, but that a large proportion (approximately 0.1 g) of Ca was obtained from the shell. They also noted that the movement of Ca appeared to occur in both directions across the yolk sac epithelium.

These findings were similar to those of Jenkins (1975) who studied the composition of a small sample of *C. novaeguineae* eggs, and concluded that most embryonic Ca was derived from the eggshell.

From these studies, it appears that prior researchers focused principally on yolk and shell and primary sources of Ca, and yolk as the primary source of P. Little mention was made of the albumen as a potential source of Ca or P for the developing embryo.

1.6.2 Regulation of embryonic Ca

Ca is maintained in homeostasis by the interaction of the hormones calcitonin, parathyroid hormone (PTH) and calcitriol (vitamin D_3). These hormones act on intestine, bone and kidney to allow excretion or promote conservation of Ca. Calcitonin lowers plasma Ca concentration by inhibiting bone resorption, promoting renal Ca excretion and reducing intestinal Ca absorption. PTH stimulates bone resorption, reduces renal excretion and promotes intestinal absorption of Ca. Calcitriol is synthesised by the liver from precursor molecules, and stimulates intestinal transport of Ca. The yolk sac epithelium, the CAM and chorioallantoic placenta (CAP) are likely targets of action for these hormones (Packard and Clark, 1996). The yolk sac is derived from the embryonic gut: therefore the physiological control of Ca transport across the yolk sac membrane may be similar to that which regulates the transport of Ca across the intestinal epithelium (Packard and Packard, 1989). Indeed, it has been confirmed that tissue of the avian yolk sac membrane expresses some of the same genes as avian intestinal epithelium, including the Ca transport genes CAT_1 and TRPV6 (Yadgary *et al.*, 2011).

Packard and Clark (1996) suggested that the yolk sac membrane is the most important site of regulation of Ca transport in species that rely heavily on yolk Ca during embryonic development (such as lizards and snakes), while the CAM may be more important among species that are rely predominantly on shell Ca (such as crocodilians).

In the embryonic chick, the CAM is derived from ectodermal, mesodermal and endodermal tissue. It functions in respiration by forming a network of capillaries lying beneath the shell, forms part of the wall of the allantoic sac containing the embryonic waste products, and actively transports water and ions to and from the embryo (Coleman and Terepka, 1972).

In the chicken egg, transport of Ca across the CAM from the shell to the foetus occurs as an energy-dependent process starting on day 12–14 of incubation and continuing until hatching

(Terepka *et al.*, 1976). This process was shown to be mediated by a Ca-binding protein and dissolution activity of the enzyme carbonic anhydrase. Interestingly, this Ca-binding protein was shown to contain residues of γ-carboxyglutamic acid, which is also found in certain Vitamin K-dependent hepatic coagulation proteins such as prothrombin. If CAM cultures were treated with the Vitamin K antagonist warfarin, the Ca transport ability of the Ca-binding protein was significantly reduced (Tuan, 1979).

Excess Ca can affect cellular function by inhibition of essential enzymes such as Na^+, K^+-activated ATPase, phosphofructokinase, pyruvate kinase and pyruvate carboxylase, and its cytoplasmic concentration is maintained within narrow limits. The cells responsible for the transport of Ca, such as those of the shell gland or intestine, are presumably equally susceptible to the negative effects of high concentrations, and therefore must perform their function without significantly changing their intracellular ionised Ca concentration (Garrison and Terepka, 1972, Packard, 1994). The process by which this occurs was partially elucidated in the early 1990s, when Akins and Tuan studied the uptake of calcium across *in vitro* cultures of ectodermal tissue using transmission electron microscopy, and by following the kinetics of a $^{45}Ca^{2+}$ marker isotope. The researchers showed that calcium taken up from the shell was likely stored in endosome-like vesicles of the chorioallantoic epithelium (Akins and Tuan, 1993a, Akins and Tuan, 1993b).

Once the chick hatches, it no longer has access to a steady supply of shell Ca and must rely on Ca in the intra-abdominal yolk for continued skeletal mineralisation until sufficient Ca can be absorbed via the gastrointestinal tract. During the late embryonic period, the yolk accumulates calcium, bound to the yolk protein phosvitin (Moran, 2007), which presumably serves as a reserve for continued bone mineralisation after hatching.

1.7 Pathologies of hatchling crocodiles

The hatching rate of *A. mississippiensis* was found to be lower in captivity than in wild populations (Joanen and McNease, 1987). The hatching rate of the Chinese alligator *A. sinensis* was low in captivity, however hatching success in wild populations was unknown (Wink and Elsey, 1994).

In captive *C. porosus*, mortality within the first year of life is a significant source of economic loss and often exceeds 15% (Webb *et al.*, 2013). Runting, also known as the failure to thrive syndrome (Huchzermeyer, 2003), is a common issue in captive crocodilian production, with a variety of suspected causes (Shilton *et al.*, 2014), including improper food or feeding strategies, social and housing-related stress, management protocols, as well as within-clutch effects (Webb *et al.*, 2013). Brien *et al.* (2014) found that clutch, but not hatchling mass at four days of age, affected the incidence of failure to thrive syndrome among *C. porosus*.

Regardless of cause, it has long been observed that hatchlings that are early to start feeding tend to be healthier than those that do not. Webb *et al.* (2013), in summarizing the work of prior researchers including Garnett and Murray (1986), stated:

> "…individual hatchlings that initiate feeding rapidly, and thus start to grow quickly, are the least likely to suffer from (failure to thrive syndrome)."

In a study of the effect of dietary variation, stocking density, handling frequency and clutch of origin on growth, feed intake and feed conversion efficiency, Garnett and Murray (1986) found that, of all the predictor variables measured, clutch of origin explained the most variability. Given that a clutch originates from a single female, it is conceivable that female-derived factors are transmitted to the offspring through the egg, which then affect the pre- and post-hatching health of the offspring. If abnormalities can be detected in the egg, perhaps they can be avoided in future clutches by treating the female appropriately prior to egg-laying.

Ca deficiency, manifesting as rickets, occurs commonly in crocodiles fed phosphorus-rich red meat without supplementary Ca, or in those kept out of sunlight without vitamin D_3 supplementation (Manolis and Webb, 2016). Some hatchlings may not be able to optimally convert the content of the egg to hatchling tissue: so that on hatching they may be nutrient deficient (Webb and Manolis, 1991).

Shilton *et al.* (2014) identified severe osteoporosis in long bones of runt *C. porosus* hatchlings, but speculated that this was likely due primarily to inanition. Serum Ca and P were found to be slightly lower in runt hatchlings than in normal hatchlings (Isberg *et al.*, 2009) however the authors suggested that this may have been related to the markedly low serum albumin of runts, since Ca is strongly albumin-bound. Total body Ca or skeletal Ca of hatchlings was not determined in these studies.

1.7.1 Pathologies of Ca and P regulation in egg formation

Halogenated hydrocarbons such as the polychlorinated biphenyls (PCBs), DDE (dichlorodiphenyldichloroethylene, $C_{14}H_8Cl_4$) and DDT (dichlorodiphenyltrichloroethane $C_{14}H_9Cl_5$) are persistent environmental pollutants that accumulate in the food chain, and have been shown to induce eggshell thinning in birds (Hickey and Anderson, 1968, Ratcliffe, 1967). The half-life of DDE is approximately 11 years (Sadasivaiah *et al.*, 2007). Despite ratifying the Stockholm Convention of 2001 (Bouwman, 2004), indoor spraying of these compounds is still done in South Africa and other African countries in an attempt to control pyrethroid-resistant malaria vectors in endemic and epidemic areas (Bouwman *et al.*, 2006). Due to its cumulative nature, species at the top of the food chain such as predatory birds, reptiles, otters and whales are vulnerable to intoxication, however sensitivity to intoxication varies among species (Lundholm, 1997).

Lundholm (1997) reviewed the known effects of these compounds on egg formation in birds. The transport of calcium across the mucosa of the shell gland is associated with the co-transport of sodium and bicarbonate ions. If the transport of either of these ions is impaired, there is likely to be a corresponding disruption in calcium transport (Lundholm, 1990). A critical step in the process of eggshell formation is the formation of carbonate ions in the shell gland, a process catalysed by carbonic anhydrase (Holm *et al.*, 2006). Bebout and Hempleman (1994) found that treatment of chickens with the carbonic anhydrase inhibitor acetazolamide decreased eggshell thickness and increased the number of shell pores per unit surface area, which greatly increased the water vapour conductance of the shell. Simply depriving the hens of dietary calcium also resulted in thinner shells, but produced no change in functional pore area. Ducks treated with *p,p'*-DDE showed significant eggshell thinning, associated with a reduction in calcium content in the lumen of the shell gland. Conversely, Stoker *et al.* (2013) found a reduced pore density in association with contamination by organochlorine compounds in *C. latirostris*, and posited this reduced pore density as a possible cause of reduced hatchability.

Disruption of prostaglandin activity represents an alternative mechanism for the toxic effects of halogenated hydrocarbons: prostaglandins may be involved in bicarbonate ion secretion in the eggshell gland. Inhibition of prostaglandin function may result in reduced bicarbonate ion secretion, which in turn would cause reduced calcium influx through the shell gland mucosa (Lundholm, 1993).

Oestrogens are required for the production of yolk and albumen, the formation of medullary bone and the mobilisation of calcium in eggshell formation in birds and reptiles (Elsey and Wink, 1986). It was hypothesised that increased hepatic clearance of oestrogens could cause thinning of shells. The mixed-function hepatic oxidase inducer, phenobarbitone was administered to laying hens, which caused a significant reduction in circulating oestradiol and calcium, together with thinner eggshells (Chen *et al.*, 1993). Exogenous compounds that lower

circulating or effective oestrogens or compete for oestrogen binding sites could conceivably result in thinner eggshells by this mechanism.

Skaare *et al.* (1991) found organochlorine residues in Nile crocodile eggs and foetuses from lakes in Kenya, but did not investigate any possible associated abnormalities. Arukwe *et al.* (2016) identified anthropogenic environmental toxins in water and Nile crocodile tissue on a South African crocodile farm, but did not specifically associate any of the toxins with identifiable egg defects. It was noted further by Arukwe *et al.* (2016) that farm management did report lower than expected hatching rates, however a variety of potential confounding factors makes the establishment of a direct causal relationship impossible. The Nile crocodile is a long-lived apex predator, and may thus be particularly susceptible to the effects of such cumulative environmental toxins which could manifest as abnormalities of egg development and shell calcification. An understanding of the pathogenesis of such conditions, together with knowledge of normal egg Ca and P distribution and metabolism could help to identify and gauge the severity of environmental intoxication.

Chapter 2. Egg processing methodology

All eggs were derived from a single crocodile farm in the North West Province, South Africa. Female crocodiles were accommodated in one of five breeder ponds. Eggs from these ponds were removed from nests by farm staff and placed in one of two incubators. A single pond contributed eggs to one incubator, and the remaining four ponds contributing eggs to the second incubator. Eggs from both incubators were used in the research detailed in this thesis. Breeder crocodiles were fed a mixture of slaughtered crocodile carcasses, and chicken carcasses enriched with a supplement containing vitamins, omega-3 fatty acids and microminerals.

In the course of this research, 967 unbanded eggs from 209 clutches were processed, together with 30 fertile eggs from 30 clutches. Unbanded eggs were collected once all fertile eggs within the clutch had hatched. Unbanded eggs (those eggs that were unfertilised, or whose embryos died at a very early stage in development) are an unfortunate by-product of captive hatchling production. However, for the researcher they have utility in that they are freely available, and are likely representative of the egg prior to consumption of any nutrients by the developing embryo or foetus.

The repeatable opening, separation, weighing and processing of many unbanded and a limited set of fertile eggs posed challenges which required invention. This chapter describes the procedures followed that were required to generate the specimens required in the research projects described in Chapters 3, 4 and 5.

2.1 Preparation prior to opening of unbanded eggs

At the processing laboratory, each uniquely numbered egg was cleaned with a gauze swab moistened with deionised water, placed on a recessed baking tray, and allowed to air dry for at least 30 min at 21 °C (Figure 2.1).

Figure 2.1.
Air drying of clean eggs

After air drying, each egg was weighed in grams using a calibrated analytical balance (Mettler-Toledo PM1200, Greifensee, Switzerland), accurate to 3 decimal places. A shield was made from a cardboard box to prevent air currents from affecting accuracy of measurement.

The greater and lesser diameter of each egg were measured in millimetres using a digital Vernier caliper, accurate to two decimal places.

Clean, dry eggs were sealed in resealable polyethylene bags, and refrigerated at 4°C prior to further processing.

2.2 Opening of unbanded eggs

To facilitate repeatable opening of eggs without cracking the calcareous eggshell, a simple lathe mechanism was designed (Figure 2.2).

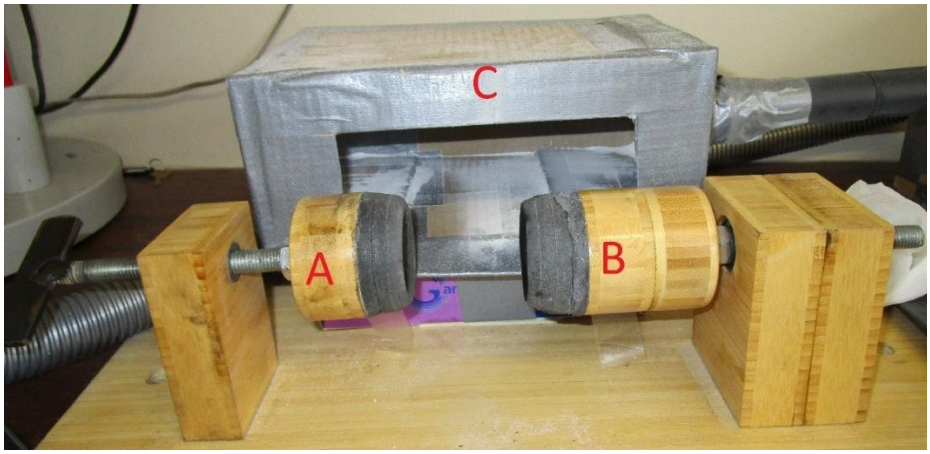


Figure 2.2.
Device for opening of eggshell. A: headstock. B: tailstock. C: vacuum dust collection port

A padded head- and tailstock (Figure 2.2, parts A and B) clamped the egg at its poles, while allowing free rotation around the long axis of the egg. A Dremel® tool (Dremel Corporation, Mount Prospect, Illinois, USA) with a diamond grit cutting wheel was used to cut through the calcareous shell around the lesser diameter, while leaving the shell membrane intact (Figure 2.3).

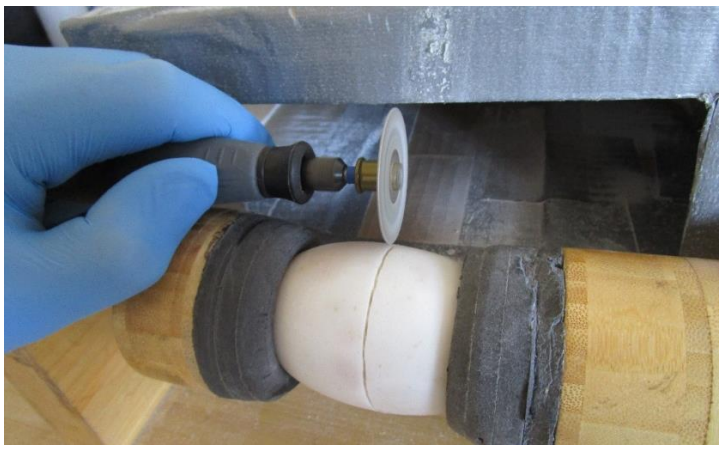


Figure 2.3.
Cutting through eggshell

Eggshell material lost as dust was collected through a cardboard port connected to a vacuum cleaner (Figure 2.2, part C). The operator of this equipment wore polycarbonate safety goggles, nitrile gloves and a facemask with an inorganic particle filter to protect against dust inhalation.

After incision with the Dremel tool, the egg was again weighed with the laboratory scale (Mettler-Toledo PM1200) to determine the quantity of shell lost as dust.

Eggs had their shell incised in batches, and were put back into resealable bags pending the following processing steps. To prevent cross-contamination, a new pair of nitrile gloves was donned for each egg during further steps in the opening process.

Using a new scalpel blade for each egg, the shell membrane visible beneath the circumferentially incised eggshell was cut (Figure 2.4).

Figure 2.4.
Incision through shell membrane

Egg content was evacuated. This required practice to avoid mixing of yolk and albumen. New disposable polystyrene fast food containers were used to contain the yolk and albumen for weighing. Each container was opened and cut in half along its hinge, and each half was marked with the egg number and “Y” for yolk, or “A” for albumen using an indelible pen. Containers were pre-weighed so that the mass of the egg component could be determined by subtraction.

With practice, it was possible to have the yolk flow out of the incised egg, while retaining the gelatinous albumen within (Figure 2.5), which could then emptied into the second container. Rotten eggs invariably had clotted albumen that could not be separated from the rotten yolk.

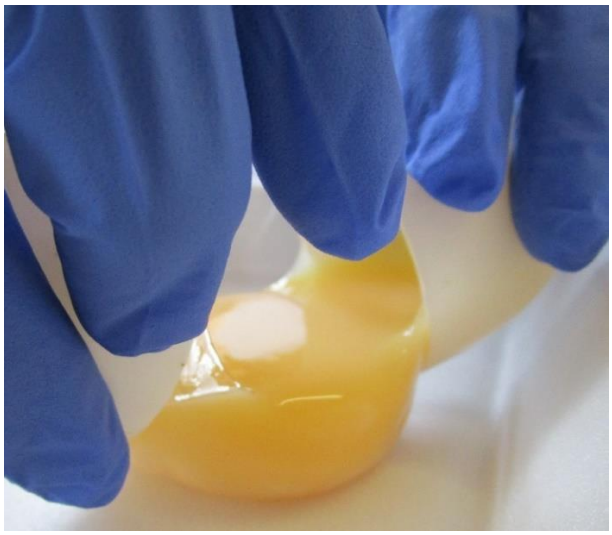


Figure 2.5.
An example of a clean separation of yolk from albumen

In many eggs, the perivitelline membrane ruptured, causing mixing of the yolk and albumen. In such cases, yolk and albumen were manually separated (Figure 2.6).

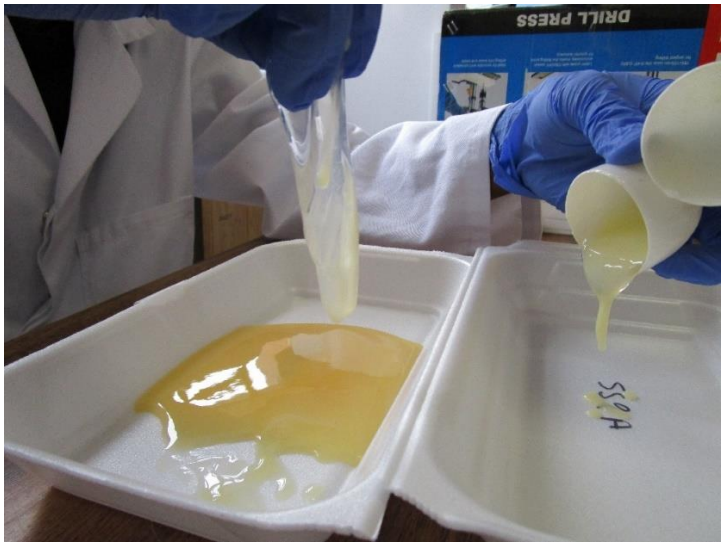


Figure 2.6.
Manual separation of yolk and albumen where the yolk was fluid

Three samples resulted from this process: a polystyrene container containing the yolk (Figure 2.7), a second polystyrene container containing the albumen (Figure 2.8), and the empty shell and shell membrane.

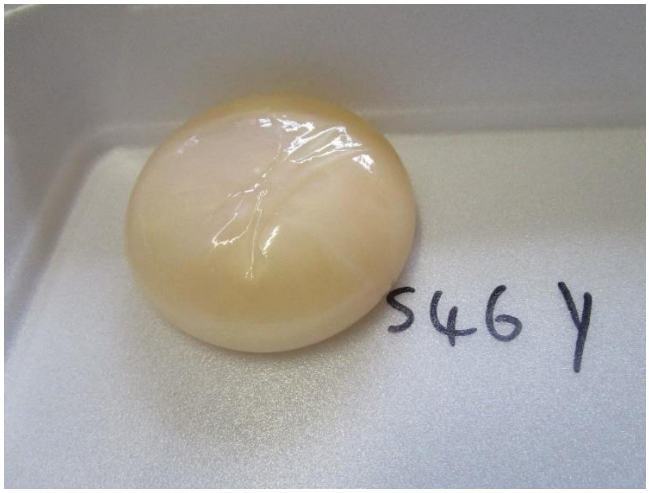


Figure 2.7.
Egg yolk with intact perivitelline membrane

Figure 2.8.
Clear uncontaminated albumen

After egg content evacuation and separation was complete, each component was weighed, and the mass of the polystyrene container was subtracted to give a figure for net component mass. The eggshell and membrane were weighed together prior to drying and separation, to obtain a combined wet weight of both these components.

Commercially available, 100 ml food-grade plastic containers were pre-weighed, marked and used for yolk and albumen frozen storage and freeze drying.

Where the perivitelline membrane was intact after egg opening and separation, it was broken using a scrap of polystyrene, and the yolk content was thoroughly mixed.

Figure 2.9.
Transfer of yolk to storage container

Approximately 30 g of yolk was then poured from the polystyrene container (Figure 2.9), into the marked, labelled yolk storage container (Figure 2.10). Residual yolk was discarded. The same was done for albumen.

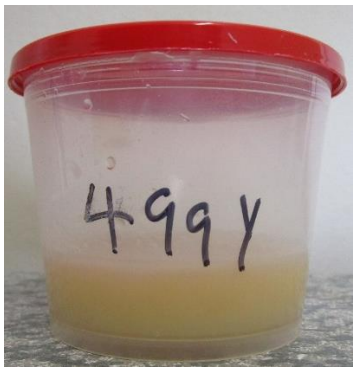


Figure 2.10.
Labelled plastic storage container, containing egg yolk

It was not always possible to perfectly separate yolk and albumen (see Figure 2.6). In some cases, this was due to variation in the liquidity of the yolk, and in other cases due to mishandling

by the operator. To quantify the degree of mixing of yolk and albumen, a clarity score was assigned.

An albumen clarity score of 1 was assigned where the albumen was clear and contained no visible contamination from yolk or other sources (Figure 2.11).

Figure 2.11.
Albumen clarity score of 1

An albumen clarity score of 2 was assigned where the albumen was mostly clear, but contained some cloudy regions where contamination by yolk or other sources had occurred (Figure 2.12).

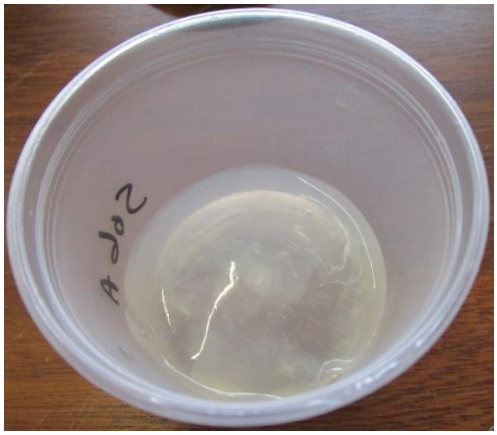


Figure 2.12.
Albumen clarity score of 2

An albumen clarity score of 3 was assigned where the albumen was heavily clouded or grossly contaminated (Figure 2.13).

Figure 2.13.
Albumen clarity score of 3

2.3 Drying of unbanded egg components

Yolk and albumen samples were freeze dried using a laboratory freeze dryer (Instruvac, Vac-Tech, Midrand, South Africa) at −50 °C and 8 mTorr in batches of 10 containers, a constraint imposed by drying chamber size. It was found through trial and error that 10 containers of approximately 30 g of yolk each, required about 36 h in the freeze dryer for desiccation, while 10 albumen containers required 72 h, presumably because of albumen's higher water content.

After weighing, the lyophilised plug of freeze-dried yolk or albumen was transferred to a labelled resealable plastic bag. The plug was crushed into a fine powder by rolling it vigorously through its resealable bag with a stainless-steel rolling pin. To ensure that yolk and albumen samples were completely free of moisture, open resealable bags were further oven dried at 50°C in a laboratory oven for an additional 12 h. In a pre-trial study, it was found that at least 4 h at 50°C were required to dry a yolk sample to constant mass. After oven drying, yolk was transferred to labelled glass vials sealed with rubber stoppers (Figure 2.14), and albumen to new labelled plastic resealable bags (Figure 2.15).

Figure 2.14.
Labelled vial containing freeze-dried and oven dried yolk

Figure 2.15.
Labelled, sealed plastic bag containing freeze dried and oven dried albumen

Once the combined wet weight of the shell and shell membrane had been determined, the empty halves of each egg's shell and shell membrane were placed in the recesses of a baking pan for drying.

Each steel baking pan could accommodate 14 empty eggshells. Baking pans were placed in a laboratory oven at 50 °C for at least six hours (Figure 2.16). It was found that no further reduction in mass of eggshell and membrane occurred after drying at 50 °C for four hours.

Figure 2.16.
Oven drying of eggshells and shell membranes

After at least six hours in the oven at 50 °C, dried shell and shell membrane were separated by hand. For most samples it was possible to simply pull the contracted shell membrane out of the shell, leaving the calcareous shell behind. In other cases, the shell membrane needed to be teased away with the edge of a scalpel blade. After separation, dried shell and membrane were placed in separate resealable plastic bags and weighed.

Shell membrane, inside its resealable bag, was crushed manually between the fingers, until it formed a relatively homogeneous collection of particles, each around 3 mm in length and width (Figure 2.17). Shell was crushed inside its resealable bag using a steel rolling pin (Figure 2.18) until it reached a maximum estimated particle diameter of 1.5 mm (Figure 2.19).

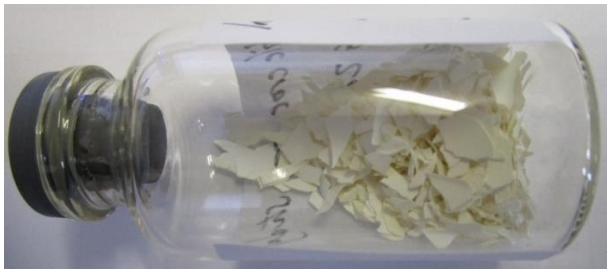


Figure 2.17.
Crushed and dried shell membrane

Figure 2.18.
Crushing of dried eggshell

Figure 2.19.
Crushed eggshell

2.4 Collection of fertile eggs

30 fertile eggs were collected, one each from 30 different clutches between the 5th of January 2017 and the 18th of January 2017, between ten and five days prior to their predicted hatching date. Working quickly, eggs were carefully removed from incubation boxes, cleaned with a gauze swab moistened in deionised water, dried with a paper towel, marked with a unique number, placed in individual resealable plastic bags and placed between frozen gel packs in a polystyrene cold storage box. Special care was taken not to subject the eggs to severe vibrations, nor to disturb neighbouring eggs in the clutch. At the processing laboratory eggs

were transferred directly to a refrigerator at 4 °C, where they remained for the next 24 h prior to further processing.

After at least 24 h in the refrigerator, the surface of each egg was cleaned again using a clean gauze swab and deionised water. They were then allowed to air dry for one hour at 21 °C. As for unbanded eggs, they were weighed on an analytical balance and dissected carefully into pre-weighed, marked polystyrene containers.

2.5 Dissection of fertile eggs

In contrast to those of unbanded eggs, the shells of late-incubation fertile eggs are thin, brittle and crack easily. The shell was tapped at the pole using the handle of a pair of surgical scissors (Figure 2.20), and shell was picked off with Adson tissue forceps (Figure 2.21), leaving the intact shell membrane behind.

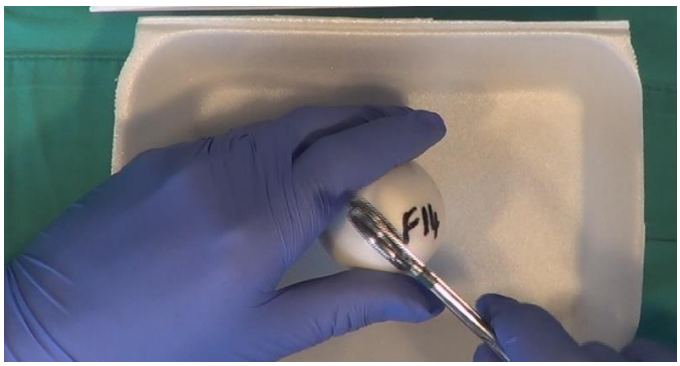


Figure 2.20.
Breaking eggshell at the pole

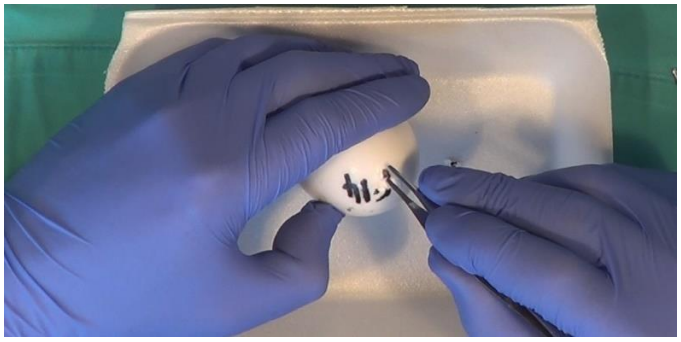


Figure 2.21.
Picking off shell fragments

Once a portion of shell approximately 3 cm in diameter had been removed from the pole of the egg, a pair of surgical scissors was used to make a cross-shaped perforation in the shell membrane, through which the foetus was extracted. This was easiest if the foetus was oriented with its head facing the hole (Figure 2.22).

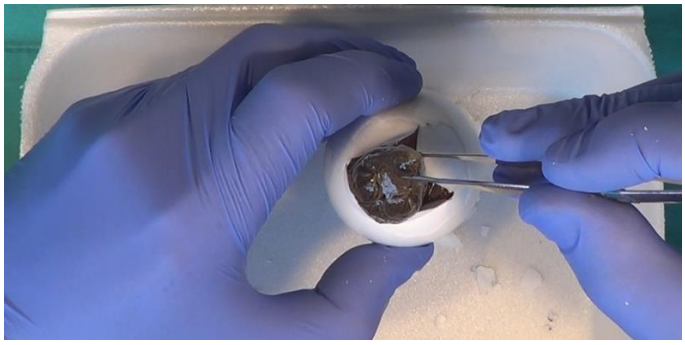


Figure 2.22.
Head-first extraction of the foetus from the egg

Where the coiled tail of the foetus was oriented towards the hole, patient manipulation allowed the tail to be uncoiled through the hole and the foetus delivered tail-first (Figure 2.23).

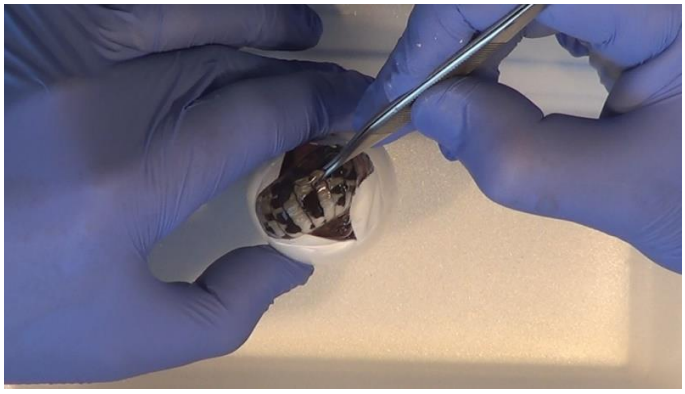


Figure 2.23.
Tail-first extraction of the foetus from the egg

After extracting the foetus from the egg, the attachment between the foetal membranes and the intra-abdominal yolk sac was cut (Figure 2.24).

Figure 2.24.
Cutting of attachment between foetal membranes and yolk sac

The foetus, together with its intra-abdominal yolk, was weighed in a polystyrene container, and the spinal cord was severed by forcing a scalpel blade between the skull and the first cervical vertebra (Figure 2.25).

Figure 2.25.
Severing of spinal cord with scalpel blade

The intra-abdominal egg yolk was removed by opening the peritoneal cavity with surgical scissors, clamping the yolk's attachment to the foetal intestine with a haemostat, and cutting the attachment with scissors (Figure 2.26).

Figure 2.26.
Separation of intra-abdominal yolk from foetal intestine

The yolk and separated foetus were then weighed in their respective polystyrene weighing containers. The yolk was transferred to a pre-weighed plastic container for freeze-drying. The perivitelline membrane surrounding the egg yolk was broken using a new scalpel blade.

The foetus was homogenised in a food processor for three minutes (Figure 2.27), together with a weighed quantity of deionised water (approximately 50 ml in volume). At the end of the three

minute period, a greyish tissue slurry remained (Figure 2.28), which was transferred to a pre-weighed plastic container for freeze-drying.

Figure 2.27.
Homogenisation of the foetus

Figure 2.28.
Homogenised foetal tissue

Remaining foetal fluid and foetal membrane within the egg were evacuated and placed in pre-weighed plastic containers for freeze drying.

Freeze drying of the homogenised foetus, yolk of the fertile egg, and the mixture of remnant foetal fluids and foetal membranes followed the same method used for yolk and albumen of

unbanded eggs. Foetal yolk, and the mixture of foetal membranes and remnant foetal fluids required 36 h to freeze dry completely, while foetal tissue required 72 h.

The wet shell and shell membrane were manually separated using a gloved finger. It was much easier to separate the shell and shell membranes of fertile eggs than those of unbanded eggs.

After separation and weighing, the shell membrane and shell were placed in separate pre-weighed plastic containers and oven-dried at 50 °C for at least six hours. Shell and shell membrane, as well as freeze dried homogenised foetus, yolk, foetal membranes with foetal fluids were then oven dried at 50 °C for at least six hours (as detailed for unbanded eggs). Dried samples were crushed within resealable plastic containers, and transferred to sealed, labelled glass vials pending analysis by a commercial laboratory (for further details of laboratory analysis, refer to Section 4.4.3).

Chapter 3. Egg mass relative to component mass in unbanded and fertile *Crocodylus niloticus* eggs

A modified version of this chapter was published in the journal *Aquaculture* with the following reference:

Brown, G.J., Forbes, P.B., Myburgh, J.G. and Nöthling, J.O., 2019. Nile crocodile (*Crocodylus niloticus*): Egg mass relative to egg component mass in unbanded and fertile eggs. *Aquaculture*, 505, pp.423-430

3.1 Abstract

Crocodylus niloticus eggs are a useful starting point to study reproduction in this species. Using samples collected from a single farm during a single breeding season, the present research aimed to describe and compare the masses of unbanded and fertile eggs and their components. The clustering effect of clutch on egg and egg component mass was investigated, and the relationship between the mass of unbanded eggs and their components, together with the effect of possible confounding variables was explored.

Estimated egg volume (ellipsoid volume) was strongly positively correlated with egg mass. A strong positive linear relationship existed between egg mass and the combined mass of the foetus and intra-abdominal yolk, as well as between egg mass and the isolated yolk-free foetal mass. If egg mass and incubation period were kept constant, foetal mass increased by 1.1 grams for each gram that yolk decreased. The wet yolk and dried shell masses of fertile eggs were significantly lower than those of size-matched unbanded eggs. Clutch had a strong clustering effect on all component masses, particularly total egg mass and hatchling mass. Unbanded egg mass and its individual component masses tended to be similar within a clutch, however some variability existed which should not be discounted.

The mass of an egg was strongly positively linearly correlated with the mass of each of its components.

The period within the laying season an egg was laid had no effect on its mass nor the mass of any of its components, whereas the breeding pond in which the female resided did affect these measurements.

The strong clustering effect of clutch on total egg mass and the masses of all egg components must be accounted for when selecting samples for future studies. The potential confounding effect of breeding pond of origin (which related to female size in the current study) should be considered, particularly where the age or size of females differ among ponds.

3.2 Keywords

Nile crocodile; clutch; egg; egg mass; yolk; shell

3.3 Introduction

The Nile crocodile (*Crocodylus niloticus* Laurenti 1768), a threatened apex predator of African freshwater ecosystems (Pooley, 1982), is intensively farmed for skin and meat in Southern Africa (Fergusson, 2010). Eggs may be collected from the nests of wild or captive females (Dzoma *et al.*, 2008, Thorbjarnarson, 1999).

Production of healthy hatchlings dictates the success of farming with crocodilians. Nelson *et al.* (2010) showed that yolk mass, as well as albumen mass, are positively related to the mass of American alligator (*Alligator mississippiensis*) eggs, although albumen mass increases more rapidly with an increase in egg mass. They also showed that the mass of *A. mississippiensis* foetuses increases in a 1:1 ratio with a decrease in yolk mass, suggesting that the yolk mass of an egg is an important determinant of hatchling mass. Working on the American alligator, Ferguson (1982) showed that part of the outer aspect of the shell is lost due to acidic dissolution from the environment, and part of the inner aspect of the shell is lost to provide Ca to the developing conceptus. Brien *et al.* (2014) showed that *Crocodylus porosus* egg mass was predictive of hatchling mass, and that the growth trajectory of hatchlings in the first 24 days of life was predictive of their growth trajectory within the first 90 days and beyond. Hutton (1987) suspected that differences in the growth rate of Nile crocodile hatchlings of similar initial size could be ascribed to differences in their yolk-free body mass and the mass of their intra-abdominal yolk. Unbanded eggs are those that do not form an opaque band around their lesser circumference, suggesting failure of fertilisation or early embryonic death. They are freely available on crocodile farms. The mean hatchling mass of clutches is positively related to the mean egg mass of clutches in various crocodilian species (Deitz and Hines, 1980, Garnett and Murray, 1986, Stoker *et al.*, 2013). These studies suggest a need to describe the size and ratios of egg components to total egg mass, and to compare the component masses between unbanded

and fertile eggs, as such information will facilitate future studies aimed at better understanding the causes of variation in the size, growth and survival of hatchlings.

Clustering effects may reduce the power of a study, and must be identified and quantified prior to study design (Killip *et al.*, 2004). The intracluster correlation coefficient (r_{ic}) quantifies the similarity of subjects within clusters with respect to a variable of interest. If r_{ic} approaches zero, there is little similarity among subjects within a cluster and if it approaches one, subjects within a cluster approach equality with respect to the variable. The gain in power of a study by including more than one subject from a cluster in a sample decreases with an increase in r_{ic}. When r_{ic} reaches one, there is no gain in including more than one subject per cluster (Dohoo *et al.*, 2009). Whitehead (1987) stated that one egg from a clutch provides a good estimate of the average egg mass of a clutch. In support of this view, for *C. porosus,* the variation of egg size among clutches is larger than that within clutches (Garnett and Murray, 1986). The same is true for *A. mississippiensis* (Deitz and Hines, 1980). These studies, as well as those of Stoker *et al.* (2013) on *Caiman latirostris* and Pooley (1962) on *C. niloticus* also show considerable variation in egg size within clutches. From this follows that there is a need to quantify the clustering effect of clutch on the size of crocodilian eggs and their components.

Flowing from the above, the four aims of this descriptive study on the Nile crocodile are as follows:

1. To quantify the mass of the components of unbanded eggs and those of fertile eggs that are within a few days of hatching, relative to egg mass.
2. To compare the component masses of unbanded and fertile eggs.
3. To quantify the clustering effect of clutch on total egg mass and the masses of the individual components of unbanded eggs.

4. To determine the relationship between the mass of unbanded eggs and that of their components, as well as to evaluate the effect of potential confounding variables on unbanded egg and component mass.

3.4 Materials and methods

The University of Pretoria Animal Ethics Committee approved the study (certificates v104-15 and v109-16 (Appendix F)).

3.4.1 Egg collection

C. niloticus eggs were collected in 2016 from a commercial farm in the North West Province, South Africa. Clutch information was copied from farm records (lay date, predicted hatching date, actual hatching date, total eggs in clutch, and number of live hatchlings).

Eggs from five breeder ponds were incubated under vermiculite in polystyrene boxes separated by clutch at 30–32 °C, 85% relative humidity.

Unbanded eggs (967 from 209 clutches) were collected once all viable eggs within a box had hatched, and fertile eggs (30 from 30 clutches) were collected between five and ten days before predicted hatching date. Each experimental egg was uniquely numbered prior to transfer to the laboratory. For transport, fertile eggs were surrounded by ice packs for at least two hours to render the foetus insensible, and then stored in a laboratory refrigerator at 4 °C for a minimum of 24 h prior to processing, which occurred within 48 h of collection.

Immediately prior to laboratory processing, all eggs were cleaned with gauze moistened with deionised water, and its surface air dried for an hour at 21 °C in a well-ventilated room. After drying, individual egg mass was determined in grams to three decimals using a calibrated analytical balance (Mettler-Toledo PM1200, Greifensee, Switzerland). Throughout the study, this is the mass used and reported as “egg mass”.

3.4.2 Processing of unbanded eggs

After cleaning, 176 “unbanded” eggs were found to in fact be banded or rotten and were discarded. The maximum length and width of each remaining unbanded egg was measured in millimetres using a digital Vernier caliper accurate to two decimal places. The formula for the volume of an ellipsoid was used to approximate volume, since crocodilian eggs are accepted to be ellipsoid in shape (Marzola *et al.*, 2015, Stoker *et al.*, 2013). Cleaned, dried eggs were placed in resealable plastic bags and refrigerated at 4 °C pending further processing. Eggs were not weighed again after refrigeration, so an unmeasured loss of mass may have occurred from transpiration during refrigeration.

A device was made to hold an unbanded egg firmly while rotating it around its long axis, without cracking the eggshell (Figure 2.2: note padded head- and tailstocks (A and B), and dust collection shroud C). The eggshell was then cut around its width with a Dremel® tool (Dremel Corporation, Mount Prospect, Illinois, USA) and a fine cutting disc leaving the underlying shell membrane intact.

After cutting the eggshell, the egg was weighed again, and the shell membrane beneath the incised shell was cut using a scalpel blade. Egg contents were evacuated, where possible avoiding mixing of yolk and albumen. Yolk, albumen, and combined shell and shell membrane were separated into disposable pre-weighed polystyrene containers for weighing, and each component’s isolated wet mass was determined by subtraction. The difference in egg mass before and after the eggshell cutting process, was added to the combined wet mass of shell and membrane, to find a total wet mass of the shell and shell membrane.

An albumen clarity score (CS) was assigned.

A CS of 1 was assigned where albumen was clear, with no visible contamination from yolk or other sources (Figure 2.11). A CS of 2 was assigned where the albumen was mostly clear, but contained some cloudy regions of contamination (Figure 2.12). A CS of 3 was assigned where the albumen was heavily clouded or grossly contaminated (Figure 2.13).

To reliably separate the shell and shell membrane of unbanded eggs, they were oven dried (Labcon, Ferndale, South Africa) at 50 °C for six to eight hours, after which they were separated and weighed into pre-weighed resealable plastic bags.

To obtain an estimated total dry mass of the unbanded eggshell, the mass of dried shell was added to a figure calculated to be the mass of dry unbanded shell lost as dust during cutting. This figure was calculated by assuming first that the proportional water content of unbanded eggshells was identical to that of fertile eggshells. The dry mass of each fertile eggshell (n = 30) was divided by its wet mass, both of which were explicitly measured. The average figure of all measurements (0.9620) was then multiplied by the wet mass of unbanded eggshell lost as dust during the Dremel cutting process, to obtain a figure for dry mass of unbanded eggshell lost during this process.

Unlike dry shell mass, dry unbanded shell membrane mass was directly measured. The respective dry masses of yolk and albumen were determined for eggs with CS of 1 (n = 185), by freeze-drying a sub-sample at -50 °C, 80 mTorr using a laboratory freeze dryer (Air and Vacuum Technologies, Midrand, South Africa) and oven-drying at 50 °C until no further loss of mass occurred over a two-hour oven-drying period. The calculated dry mass proportion from the sub-sample of each component was then multiplied by the total wet mass of that component to determine the dry mass fraction of the entire component.

3.4.3 Fertile eggs

For each fertile egg (n = 30), the mass of the foetus, the foetal membranes together with foetal fluid, the yolk, the shell and the shell membrane were directly measured.

Shells of fertile eggs in late incubation are thin and brittle, and were opened by tapping the eggshell at one of the poles and picking off shell fragments, leaving intact shell membrane behind. Once a portion of shell roughly 3 cm in diameter had been removed, the shell membrane was perforated, the foetus removed and the attachment between foetal membranes and intra-abdominal yolk sac cut.

Killing of foetuses within fertile eggs was performed according to IUCN-CSG recommended practices (Manolis and Webb, 2016). By the time each foetus was removed from its egg, it had been kept at or below 4 °C for at least 24 h. To ensure that it was dead and not merely in a state of torpor, the foetus was immediately decapitated: no haemorrhage or movement occurred during this process.

The abdominal wall was incised, and the yolk contained in the perivitelline membrane was removed from the abdomen. The separated yolk and foetal tissue were then weighed, and the foetus was homogenised. After removing the foetus, a mixture of foetal fluid, foetal membranes and remnant albumen remained within the egg. These were removed and weighed together.

Unlike those of unbanded eggs, the shell and shell membrane of fertile eggs could be easily separated when wet. The separated wet masses of eggshell and shell membrane were measured. Once separated, all fertile egg components were freeze- and oven-dried, and their dry masses measured.

3.4.4 Data analysis

Microsoft Access and Excel (Microsoft Corporation, Redmond, Washington, USA) were used for data storage and manipulation, and Stata 14 (StataCorp LP, College Station, Texas, USA) was used for statistical analysis.

3.4.5 Description of the masses of egg components and hatchlings relative to total egg mass (aim 1 of the study)

3.4.5.1 Masses of unbanded eggs and their components

The mass contribution of each component to the total mass of each unbanded egg was calculated and, ignoring clutch, the mean, standard deviation, minimum and maximum were reported.

The relationship between egg mass and ellipsoid egg volume was determined by simple regression.

3.4.5.2 Masses of fertile eggs and their components

The mass contribution of each component to the total mass of each fertile egg was calculated and, ignoring clutch, the mean, standard deviation, minimum and maximum reported.

Linear regression was used to determine the relationship between foetal mass and egg mass, foetal mass and yolk mass and yolk mass and egg mass. Multivariable regression was used to determine the effects of egg mass, yolk mass and incubation length on foetal mass, and to compare component masses of fertile and unbanded eggs.

3.4.6 Comparing component masses of unbanded and fertile eggs (aim 2 of the study)

For each fertile egg, one CS 1 unbanded egg was selected such that the masses of the two were as close as possible to equal to form a mass-matched pair. A one-tailed paired t-test was used

to determine whether the yolk mass and dry shell mass of fertile eggs were respectively lower than those of unbanded eggs.

3.4.7 Quantifying the clustering effect of clutch on the masses of unbanded eggs and their components (aim 3 of the study)

The clustering effect of clutch was quantified by determining the r_{ic} with clutch as cluster variable for the masses of unbanded eggs and their components. To determine the r_{ic} for each of these variables, no covariates were included in the model and only those clutches were used for which the masses of at least two unbanded eggs or their components were available.

The within-clutch ranges in mass of wet unbanded eggs and their components were determined to demonstrate the variability within clutches.

3.4.8 The relationship between the mass of unbanded eggs and their components, as well as the effect of potential confounding variables on unbanded egg and component mass (aim 4 of the study).

Selected variables that could confound the masses of eggs and their components were assessed, as such confounders must be accounted for in the design of future studies. Using clutch as a cluster variable, a multilevel mixed-effects regression model was used to assess the effect of egg mass and the potential confounding covariates of pond, albumen clarity score, date of laying, number of eggs in clutch and within-clutch percentage of fertile eggs on egg mass, wet yolk mass, combined wet mass of shell and shell membrane, dry shell mass, and dry shell membrane mass of unbanded eggs. Covariates that had no significant effect on the outcome variable ($P \geq 0.05$) were sequentially removed from the model.

3.5 Results

3.5.1 Masses of unbanded eggs and their components

Of 791 unbanded eggs from 198 clutches, 185 from 95 clutches had an albumen clarity score (CS) of one, 291 from 138 clutches had a CS of two and 315 from 150 clutches had a CS of three. A summary of the masses and mass ratios of the components of unbanded eggs are presented in Table 3.1 and Table 3.2. All variables were positively skewed, with the exception of wet albumen mass, which was negatively skewed.

Table 3.1.
Summary of the masses (in grams) of unbanded eggs and their components, ignoring the clutch of origin of each egg or egg component

Component	Mean	SD	Min	Max	n
Egg mass	97.76	16.39	57.65	144.81	791
Combined wet shell and membrane	14.61	2.36	9.41	22.68	791
Dry shell	10.50	1.70	6.75	16.00	791
Dry shell membrane	1.52	0.26	0.96	2.53	791
Wet yolk	39.63	9.07	21.59	74.03	185
Wet albumen	36.32	7.06	17.75	54.80	185
Dry yolk	26.11	5.00	16.06	37.42	101
Dry albumen	15.55	3.77	8.35	29.78	101

Table 3.2.
Masses of unbanded egg components expressed as a percentage of total egg mass, ignoring the clutch of origin of each egg or egg component

Component	Mean %	SD %	Min %	Max %	n
Combined wet shell and membrane	15.01	1.21	12.12	21.78	791
Dry shell	10.79	0.91	8.57	14.31	791
Dry shell membrane	1.57	0.19	1.07	2.45	791
Wet yolk	42.56	5.61	29.44	62.21	185
Wet albumen	39.24	4.99	21.32	49.41	185
Dry yolk	28.13	3.34	21.97	38.39	101
Dry albumen	16.89	3.94	8.08	35.05	101

If eight extreme values were removed (representing likely transcription or typographical errors), a strong positive linear relationship was evident between ellipsoid egg volume and egg mass (Figure 3.1), with the equation:

Volume in millilitres = 0.26 + 0.88 (egg mass in grams)

(SE of coefficient of egg mass 0.003, 95% CI 0.87–0.89, $r^2 = 0.99$, n = 784, df = 782, $t = 255.4$, $P < 0.001$)

Intuitively, the gradient of the fitted line approximates egg density.

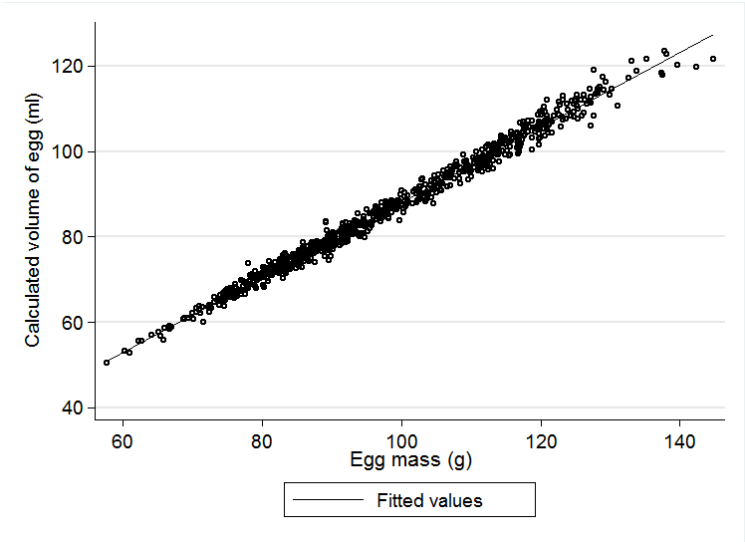


Figure 3.1.
Relationship between egg mass and volume, calculated as the volume of an ellipsoid

3.5.2 Masses of fertile eggs and their components

A summary of the masses of fertile egg components and their mass ratios are shown in Table 3.3 and Table 3.4.

Table 3.3.
Summary of fertile egg and component masses, expressed in grams

Component	Mean	SD	Min	Max	n
Egg mass	105.67	12.50	78.86	124.31	30
Wet shell	11.07	1.09	8.54	13.79	30
Wet shell membrane	3.39	0.36	2.75	4.12	30
Dry shell	10.66	1.10	8.18	13.59	30
Dry shell membrane	1.54	0.15	1.12	1.90	30
Wet yolk	17.62	4.75	11.19	30.28	30
Wet foetus	50.39	7.72	36.06	68.29	30
Wet foetal membranes	21.53	4.17	16.10	30.56	30
Dry yolk	7.82	2.15	5.16	13.05	30
Dry foetus	23.16	2.88	16.49	28.55	30
Dry foetal membranes	0.84	0.50	0.42	2.22	30

Table 3.4.
Masses of fertile egg components expressed as a percentage of total egg mass

Component	Mean (%)	SD (%)	Min (%)	Max (%)	n
Wet shell and shell membrane [a]	13.79	1.34	10.77	16.56	30
Wet shell	10.56	1.13	8.55	12.94	30
Wet shell membrane	3.24	0.38	2.23	3.99	30
Dry shell	10.17	1.14	8.10	12.70	30
Dry shell membrane	1.47	0.15	1.17	1.72	30
Wet yolk	16.57	3.25	10.46	24.36	30
Wet foetus	47.77	5.52	32.85	58.49	30
Wet foetal membranes	20.36	2.93	14.94	28.44	30
Dry yolk	7.35	1.48	4.88	10.57	30
Dry foetus	22.12	3.22	17.42	29.26	30
Dry foetal membranes	0.81	0.49	0.39	2.33	30

[a] Determined by addition of wet shell and wet membrane

A single foetus with a broken yolk, that caused a non-normal distribution of the residuals and heteroskedasticity, was removed from the model assessing the relationship between the combined mass of the foetus and its intra-abdominal yolk as well as other models involving yolk and foetus.

There was a strong positive linear relationship between egg mass and the combined mass of the foetus and its intra-abdominal yolk (Figure 3.2a). The relationship between the combined mass of the foetus and its intra-abdominal yolk, with incubation period as covariate is described by the following regression equation:

Mass of foetus with its intra-abdominal yolk = −39.4 g + 0.50 (incubation period) + 0.70 (egg mass). The F-statistic (2, 26 df) = 90.2, $P < 0.001$, $r^2 = 0.87$.

Keeping egg mass constant, the combined mass of the foetus and its intra-abdominal yolk increased by 0.50 grams for every day that the incubation period increased (SE 0.24, 95% CI 0.01–1.00, n = 29, df = 26, $t = 2.08$, P = 0.047). Keeping incubation period constant, the mass of the foetus with its yolk increased by 0.70 g for every increase by one gram in egg mass (SE 0.055, 95% CI 0.59–0.82, n = 29, df = 26, $t = 12.74$, $P < 0.001$).

Figure 3.2b shows a looser positive, linear association between egg mass and the mass of the intra-abdominal yolk of the foetus in the final 10 days of incubation. Incubation period, which had no significant effect (P = 0.16) was removed from the model. The regression equation was:

Yolk mass = −9.75 + 0.26 (fertile egg mass). The F-statistic (1, 27 df) = 21.9, $P < 0.001$, $r^2 = 0.45$.

(SE = 0.06, 95% CI = 0.15–0.37, n = 29, df = 27, $t = 4.68$, $P < 0.001$)

The yolk-free isolated foetal mass was significantly linearly associated with egg mass, but with substantial variation (Figure 3.2c). Incubation period, which had no significant effect (P = 0.67) was removed from the model. The regression equation was:

Isolated foetal mass = 2.15 + 0.46 (egg mass). The F-statistic (1, 27 df) = 34.1, P < 0.001, $r^2 = 0.56$.

(SE = 0.08, 95% CI = 0.30–0.63, n = 29, df = 27, *t* = 5.84, P < 0.001)

No significant association existed between the mass of the intra-abdominal yolk, and the wet mass of the foetus from which it was removed (P = 0.40).

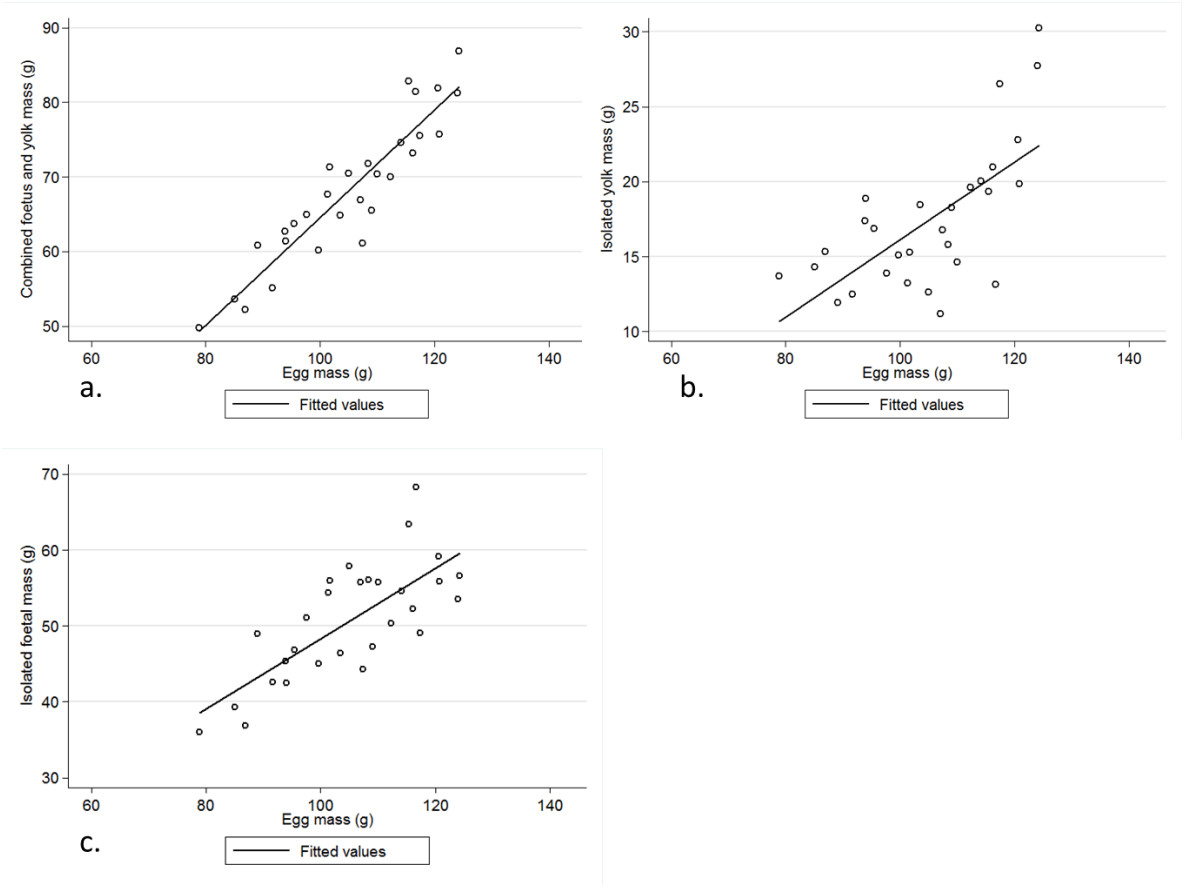


Figure 3.2.
Association between total egg mass and (a) combined mass of foetus and intra-abdominal yolk, (b) intra-abdominal yolk, and (c) and yolk-free foetus

The regression equation describing the effects of incubation period, egg mass and the mass of intra-abdominal yolk on isolated yolk-free foetal mass was as follows:

Isolated yolk-free foetal mass = −42.47 g + 0.54 (incubation length) + 0.73 (egg mass) – 1.10 (yolk mass). The F-statistic (3, 25 df) = 33.6, P < 0.001, r^2 = 0.80.

Isolated yolk-free foetal mass increased by on average 0.54 g for each incubation day between ten and five days prior to predicted hatching date (SE = 0.26, 95% CI = 0.01–1.06, n = 29, *t* = 2.10 (25 df), P < 0.05). Keeping the incubation period and yolk mass constant, the isolated yolk-free foetal mass increased by 0.73 g for each increase in egg mass by one gram (SE = 0.07, 95% CI 0.57–0.88, n = 29, *t* = 9.76 (25 df), P < 0.001). Keeping egg mass and incubation period constant, isolated yolk-free foetal mass increased by 1.1 g for each gram that the yolk decreased (SE = 0.20, 95% CI = −1.51 to −0.68, n=29, *t* = −5.49 (25 df), P < 0.001).

3.5.3 Comparison between component masses of unbanded and fertile eggs

The egg masses of the unbanded and fertile eggs of each of the 29 mass-matched pairs differed by no more than 5.0 g.

The wet yolk mass of the 29 fertile eggs was 28.64 g (SE 1.28, 95% CI 25.81–31.48) lower than those of the 29 unbanded eggs (t = 20.7, df = 28, P < 0.001), and their dried shell mass 0.49 g (SE 0.27, 95% CI −.06 to 1.04) lower (t = 1.82, df = 28, P = 0.04) (Figure 3.3).

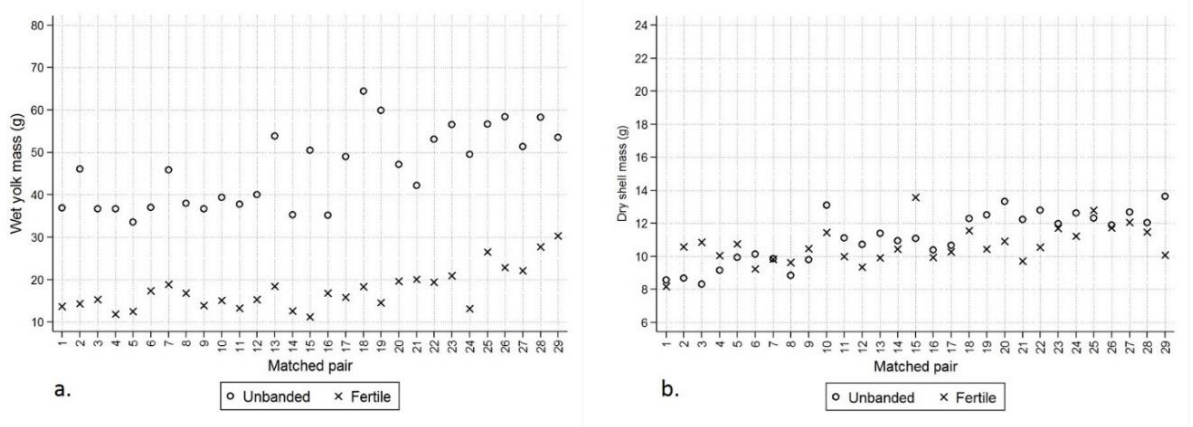


Figure 3.3.
Comparison of masses of wet yolk (a) and dry shell (b) for fertile and unbanded size-matched eggs

3.5.4 The clustering effect of clutch on the masses of unbanded eggs and their components

Clutch had a strong clustering effect on the mass of all egg components, particularly total egg mass and dry shell mass (Table 3.5). The high r_{ic}'s for the mass of unbanded eggs and the components thereof suggest a high degree of similarity of these respective masses within clutches. The r_{ic}'s indicate that 90% of the variation in egg mass, 89% of the variation in dry shell mass, 74% of the variation in dry shell membrane mass, 79% of the wet yolk mass and 66% of wet albumen mass are due to variation among clutches. They also indicate that 10% of the variation in egg mass, 11% of the variation in dry shell mass, 26% of the variation in dry shell membrane mass, 21% of the wet yolk mass and 34% of wet albumen mass are due to variation within clutches.

Of those 185 clutches from which the mass of two or more unbanded eggs were collected, the mean egg mass of the clutches varied from 63.7 g to 139.0 g, whereas the mean and SD of the clutch means were 98.1 g and 15.9 g. In line with the high intraclass correlation coefficient, the mean of the within-clutch SDs of these 185 clutches was 4.2, which is about one quarter of size of the SD of clutch means.

The within-clutch range of the mass of unbanded eggs and various of their components are summarised in Table 3.6, which shows that egg mass varied by as little as 0.06 g in one clutch and by as much as 57.0 g in another, whereas egg mass varied by 8.1 g or less in half the clutches and by 8.1 g or more in the remaining half. When the 185 clutches are sorted in order of increasing range of their egg masses, the range in egg mass within clutches varied by 6.8 g among the middle 92 of the clutches. When viewed against the overall mean egg mass of 98 g, these small ranges within clutches are in line with the very high intraclass correlation coefficient for egg mass. Figure 3.4 and Figure 3.5 show large variations in wet yolk mass and dry shell mass among clutches relative to substantially smaller variations within clutches,

which are in line with the high intraclass correlation coefficients shown in Table 3.5 and the relatively small within-clutch ranges shown in Table 3.6.

Table 3.5.
Intraclass correlation coefficients (ric) of unbanded Nile crocodile eggs, measured for clutches containing two or more sampled unbanded eggs

Measurement	Specimens	Clutches	r_{ic}
Egg mass	779	186	0.90
Dry shell mass	779	186	0.89
Dry shell membrane mass	779	186	0.74
Wet yolk mass (CS 1)	92	46	0.79
Wet albumen mass (CS 1)	182	92	0.66

Table 3.6.
Summary of within-clutch ranges in the masses of unbanded eggs and their components

	Range in mass (g)				
Component	Min	Median	Maximum	IQR	Clutches
Egg	0.06	8.06	56.97	6.80	185
Wet yolk (CS 1)	0.05	4.74	17.12	4.73	46
Dry shell (CS 1)	0.01	0.46	1.95	0.62	46

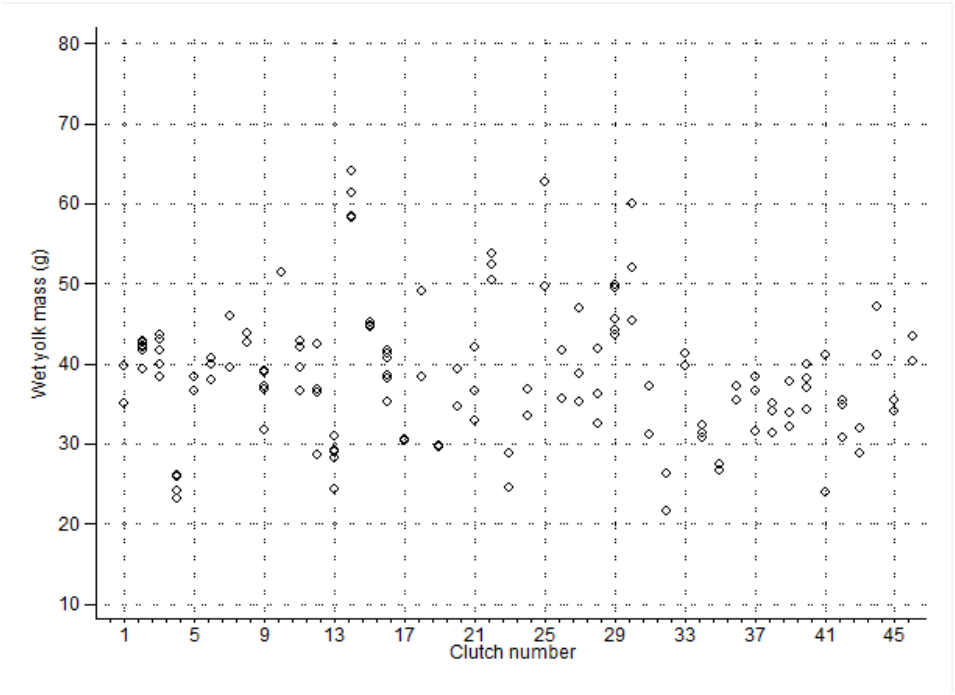


Figure 3.4.
Wet yolk mass of each egg in each of 46 clutches containing at least two eggs with a clarity score of one

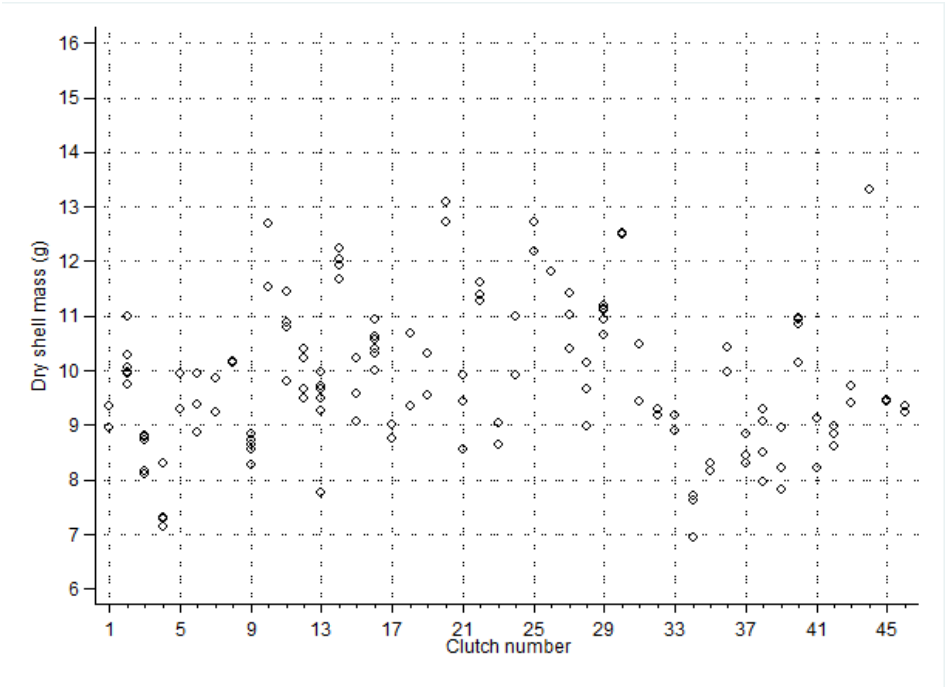


Figure 3.5.
Dry shell mass of each egg in each of 46 clutches containing at least two eggs with a clarity score of one

3.5.5 Relationship between the mass of unbanded eggs and their components, and the effect of potential confounding variables

3.5.5.1 The association between potential confounders and egg mass

Neither date of laying (P = 0.75) nor within-clutch hatching percentage had any effect on egg mass and they were removed from the model. Pond and number of eggs per clutch remained in the final model (n = 792 eggs, 198 clutches, r^2 = 0.55, Wald Chi-squared (5 df) = 259.5, P < 0.001). Keeping the number of eggs per clutch constant, females in pond 3 laid eggs that were on average 22.2 g heavier than females in pond 0 (SE 2.33, 95% CI 17.6–27.6, *z* = 9.50, P < 0.001) and females in pond 4 laid eggs that were on average 22.2 g heavier than eggs laid by females in pond 0 (SE 2.16, 95% CI 18.0–26.5, *z* = 10.28, P < 0.001). Keeping pond constant, egg mass decreased by 0.20 g for each increase by one in clutch size (SE = 0.08 g, 95% CI −0.37 to −0.05, *z* = −2.51, P = 0.01).

3.5.5.2 The association between potential confounders and wet yolk mass

Neither within-clutch percentage of fertile eggs (P = 0.18) nor date of laying (P = 0.14) nor pond (P > 0.10) nor number of eggs per clutch (P = 0.25) affected wet yolk mass, and they were removed from the model. Egg mass remained in the final model (n = 185 eggs, 95 clutches, r^2 = 0.67, Wald Chi-squared (1 df) = 224.4, P < 0.001). The final regression equation was:

Wet yolk mass = −6.68 + 0.50 (egg mass).

For each additional gram that egg mass increased, wet yolk mass increased by 0.5 g (SE = 0.03, 95% CI 0.44–0.57, *z* = 14.98, P < 0.001).

3.5.5.3 The association between potential confounders and the mass of the shell and shell membrane

Neither within-clutch percentage of fertile eggs (P = 0.92) nor date of laying (P = 0.56) nor number of eggs per clutch (P = 0.44) affected the wet mass of the shell and shell membrane combined and they were removed from the model. Egg mass, pond and clarity score of the albumen (CS) remained in the final model (n = 792 eggs, 198 clutches, $r^2 = 0.77$, Wald Chi-squared (7 df) = 1326, P < 0.001).

Controlling for pond and CS, the combined wet mass of shell and shell membrane increased by 0.12 g for each gram that egg mass increased (SE = 0.004, 95% CI 0.11–0.13, *z* = 27.5, P < 0.001). Controlling for egg mass and CS, the combined wet masses of shells and shell membranes of eggs laid by females in pond 3 were on average 0.48 g greater than that of eggs laid by females in pond 1 (SE = 0.24, 95% CI = 0.01–0.94, *z* = 2.01, P < 0.04). Controlling for egg mass and pond, the combined wet masses of shells and shell membranes of CS 3 eggs were on average 0.44 g greater than those of CS 1 eggs (SE = 0.09, 95% CI = 0.27–0.60, *z* = 5.1, P < 0.001).

Neither number of eggs per clutch (P = 0.53) nor within-clutch percentage of fertile eggs (P = 0.15) nor date of laying (P = 0.07) affected the dry mass of the shell and they were removed from the model. Only egg mass, pond and CS remained in the final model (n = 791 eggs, 198 clutches, $r^2 = 0.76$, Wald Chi-squared (7 df) = 1471, P < 0.001). Controlling for pond and CS, dry shell mass increased by 0.09 g for each gram that egg mass increased (SE = 0.003, 95% CI 0.08–0.09, *z* = 32.5, P < 0.001). Controlling for egg mass and CS, the dry masses of shells of eggs laid by females in pond 3 were on average 0.09 g greater than that of eggs laid by females in pond 1 (SE = 0.003, 95% CI = 0.08–0.09, *z* = 32.5, P < 0.001). Controlling for egg mass and pond, the dry shell mass of CS 3 eggs was on average 0.12 g higher than that of CS 1 eggs (SE = 0.05, 95% CI = 0.03–0.21, *z* = 2.61, P < 0.009).

Neither the number of eggs per clutch (P = 0.94) nor within-clutch percentage of fertile eggs (P = 0.51) affected dry shell membrane mass and they were removed from the model. Egg mass, pond, date of laying and CS remained in the final model (n = 791 eggs, 198 clutches, r^2 = 0.58, Wald Chi-squared (8 df) = 515, P < 0.001). Controlling for the other covariates, dry shell membrane mass increased by 0.01 g for each gram that egg mass increased (SE = 0.0007, 95% CI = 0.010–0.013, *z* = 17.67, P < 0.001). Controlling for other covariates, the dry shell membrane masses of eggs laid by females in pond 2 are expected to be 0.08 g lower than that of eggs laid by females in pond zero (SE = 0.028, 95% CI = −0.13 to −0.023, *z* = −2.80, P = 0.005). Controlling for other covariates, dry shell membrane masses are expected to decrease by 0.002 g for each day later in the season a clutch is laid (SE = 0.0009, 95% CI = −0.005 to −0.0009, *z* = −2.99, P = 0.003). Controlling for other covariates, the dry shell membrane masses of CS 3 eggs are expected to be 0.04 g higher than those of CS 1 eggs (SE = 0.014, 95% CI = −0.015 to −0.068, *z* = 3.08, P = 0.002).

3.6 Discussion

The current study benefitted from a large sample of captive-laid unbanded *C. niloticus* eggs, and a smaller sample of fertile eggs within a few days of hatching. This allowed for a comprehensive description of the size and masses of unbanded and fertile eggs and their components, as well as the between- and within-clutch variability of the masses of unbanded eggs and their components.

Deeming and Ferguson (1989) found that the mass of late-incubation alligator embryos had a positive linear relationship with initial egg mass. This finding was supported by data from the present study, where the combined wet mass of foetus and yolk depended substantially on total egg mass, with heavier eggs yielding heavier foetuses and yolks in a strong linear fashion.

The substantial difference in yolk mass between size-matched fertile and unbanded eggs confirms findings by Webb *et al.* (1987) for *Crocodylus johnstoni*. The present study's finding that the yolk-free foetal mass increases by 1.1 g for each gram that the yolk gets lighter is very similar to that of Nelson *et al.* (2010) who showed that the yolk mass of American alligator eggs decrease in a 1:1 ratio with an increase in foetal mass. If the relationship between foetal mass and yolk mass is considered in light of the large variation in the contribution of yolk to total egg mass in unbanded eggs, it follows that variation in yolk size for a particular egg mass could result in a variation in the mass of hatchlings from eggs of the same mass.

The lower dry shell mass of fertile eggs than those of mass-matched unbanded eggs is most likely due to a withdrawal of inorganic elements, principally calcium, from the eggshell as shown in the American alligator by Ferguson (1982).

The present study found a mean combined wet mass proportion of shell and membrane for *C. niloticus* that was noticeably higher than that reported by Webb *et al.* (1987) for *C. johnstoni* and *C. porosus.*

The current study shows that there is a very strong, positive, linear relationship between the masses of Nile crocodile eggs and their ellipsoid volumes, suggesting very little variation in density among eggs of different masses.

Although others have observed that the masses of crocodilian eggs are very similar within clutches (Deitz and Hines, 1980, Brien *et al.*, 2014), the current study quantified the variation within and among clutches using the intracluster correlation coefficient.

Pooley (1962) noted that there was substantial variation in length and weight of *C. niloticus* embryos from the same clutch, but did not provide figures for their masses. Garnett and Murray (1986) found that variability in egg and hatchling mass within *C. porosus* clutches was small relative to that between clutches, and provided clutch means and standard deviations. Stoker *et*

al. (2013) provided clutch means and standard errors of the means for egg masses of eight clutches of *C. latirostris*: when converted to standard deviation, the within-clutch variation was substantial.

Clutch had a very strong clustering effect on the masses of all egg components, evidenced by the high values for r_{ic}, especially for egg mass. This is of importance when planning new studies. Unless the aim of a study is to further investigate within-clutch effects on egg size, an experimental design using fewer (even only one) eggs per clutch but from as many clutches as possible would be preferred over a design that includes more eggs from fewer clutches.

Date of laying within a season had no effect on total egg mass, nor on the masses of individual components, a finding that was in agreement with that of Brien *et al.* (2014) for *C. porosus*, but different to that of Staton and Dixon (1977) for *Caiman crocodilus*.

The present study's findings suggest that the period within the laying season does not have to be considered when collecting *C. niloticus* eggs for future studies involving the masses of egg or their components, however in light of recent findings by Brien *et al.* (2014), consideration should be given to potential effects of season on other outcomes such as hatchling growth rate or the incidence of failure to thrive.

Maternal genotype, age, size, disease status and nutritional state could affect the mass of eggs and their components (Andrews, 2004). Factors which affect the size of the eggs and their components may be correlated to factors (such as size and age of the female) that could affect her probability of being bred by a fertile male, which would directly impact hatching rate. In the present study, neither egg mass nor the masses of egg components were related to hatching rate. Again, the captive environment may have masked such a relationship: a small territory inhabited by many crocodiles, with an artificially high male:female ratio may have resulted in a greater likelihood of being mated.

Eggs from ponds 3 and 4 were substantially heavier than those from pond zero. These two ponds accommodated a greater proportion of bigger, older females. The positive association between egg size and size of female crocodilians has previously been reported by Thorbjarnarson (1996) and Verdade (2001).

It cannot be explained why eggs with albumen clarity scores of 3 were not only associated with significantly heavier combined wet shell and shell membrane masses but also dry shell mass. Clarity score was independent of total egg mass.

This study provides detailed data on the variability of crocodile egg components and their association with hatchling mass. This new information will assist in the planning of further studies regarding parameters influencing captive breeding success rates.

3.7 Acknowledgements

The authors would like to thank Mr Stefan van As and Ms. Karon Griffiths for provision of sample material and assistance during data collection, and Mr Kgomotso Sekwele for assistance in egg processing.

3.8 Sources of funding

Funding for this research was provided by University of Pretoria in the form of a bursary to the first author, and by the South African National Research Foundation (NRF) in the form of an incentive fund for rated researchers to the last author (grant number 85836).

3.9 Declaration of conflicts of interest

None

Chapter 4. Calcium and phosphorus in unbanded eggs of the Nile crocodile (*Crocodylus niloticus*)

An article based on this chapter was submitted in altered form to the journal *Aquaculture Research* in June 2019.

4.1 Abstract

The Nile crocodile is farmed for leather and meat in South Africa by producing hatchlings from captive-laid eggs. Little is known about factors affecting egg hatchability and hatchling vigour. Egg provision of calcium (Ca) and phosphorus (P) could conceivably influence foetal or hatchling health. Unbanded eggs are those which do not form an opaque band around their lesser circumference, indicating either a failure of fertilisation, or early embryonic death. The present research aimed to describe the concentration and content of Ca and P in each unbanded egg component (shell, shell membrane, albumen and yolk), and to assess whether clutch had a grouping effect on the concentration or content of Ca or P in these components. Additionally, it aimed to quantify the effect of the potential confounding variables clutch size, laying date, pond of origin, component mass and component Ca and P concentration on the content of Ca or P in unbanded egg components.

Ca and P concentrations in specimens of unbanded eggs were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES), and Ca and P content was calculated from each component's respective dried mass.

Shell Ca and yolk Ca and P content were influenced primarily by the mass of their respective component, and secondarily by the concentration of each respective element. Shell membrane and albumen Ca and P were influenced primarily by the concentration of the element within the component. Shell Ca concentration was similar to that of pure calcium carbonate and was not significantly influenced by the grouping effect of clutch.

Eggshell made by far the greatest contribution to total egg Ca while contributing no measurable P.

Shell membrane was found to be a highly variable specimen type: it was suspected that this reflected issues with sample processing. Albumen contributed variable, but generally very low

quantities of Ca and P to the egg. Yolk contributed by far the greatest quantity of P and a significant quantity of Ca. Both yolk Ca and P concentration and content were substantially influenced by the grouping effect of clutch.

In the newly laid egg, the principal reservoirs of Ca for foetal development are the shell and yolk, and of P, the yolk. If cost of chemical analysis is a consideration, future studies may be advised to take specimens of only these components when evaluating the effect of preventive or therapeutic interventions.

4.2 Keywords

reptile, Nile crocodile, crocodile, crocodilia, egg, calcium, phosphorus, shell, shell membrane, albumen, yolk

4.3 Introduction

Crocodilians are grown for their skin and meat in subtropical and tropical regions throughout the world. In Southern Africa, the indigenous Nile crocodile (*Crocodylus niloticus*) is farmed intensively (Carruthers, 2008, Fergusson, 2010). Farmed hatchlings are produced from artificially incubated eggs, which are either harvested from wild nests or are laid by captive females (Thorbjarnarson, 1999). Production of vigorous hatchlings from captive-laid eggs has obvious economic benefits for the farmer.

Joanen and McNease (1977) found that *Alligator mississippiensis* eggs from wild nests had higher hatching rates than those laid by captive females. Khosa *et al.* (2012) found no significant difference in hatching rate between wild and captive-laid *C. niloticus* eggs, but found that wild-harvested clutches from certain regions of Zimbabwe contained more eggs than those laid in captivity. These findings suggest that the hatchling yield of captive-laid eggs may be lower than that of wild-harvested eggs.

Factors influencing clutch size, fertility and hatching rate in crocodilians are poorly understood. Crocodilian eggs that fail to develop, either due to infertility or due to early death of the embryo, can be identified by their failure to form a visible opaque band around their lesser circumference. Unbanded eggs are of no value to crocodile farmers but present an opportunity for researchers to study egg composition and associated influences.

Lance *et al.* (1983) suggested that maternal dietary micro- or macronutrient or fatty acid composition may influence alligator egg hatching rate. Inadequate provision or pathological metabolism of Ca and P are common in captive reptiles (Marcus, 1981). The symptoms of metabolic bone disease in crocodile hatchlings have been reviewed by Huchzermeyer (2003) and include kyphoscoliosis and rubbery jaws with glassy teeth. Lane *et al.* (1984) reported

easily fractured long bones and spinal compression fractures in alligator hatchlings fed a Ca-deficient diet on a Florida farm.

Embryos of birds and oviparous reptiles obtain Ca from yolk and eggshell (Simkiss, 1991), but the ratio of each component's contribution varies according to species (Stewart and Ecay, 2010, Packard and Packard, 1989). The role of Ca and P (or deficiencies thereof) in the suboptimal production of hatchlings by apparently healthy female crocodiles has not been well studied.

To optimise selection of sample type, egg number and clutch number in future studies, the concentration, content and variability of Ca and P in each egg component must be measured and statistically described, both within and among clutches. Additionally, the effect of potential confounding variables in sample collection must be accounted for.

Jenkins (1975) researched the mineral content of a small sample of unbanded and fertile *Crocodylus novoguinae* eggs, and Packard and Packard (1989) investigated the mobilisation of Ca and P by *A. mississippiensis* embryos, however subsequent this work the topic remains largely unresearched.

Given this gap in knowledge, the present research had the following two aims:

1. To describe the concentration and content of Ca and P in each component of unbanded Nile crocodile eggs and assess whether clutch had a significant grouping effect on these parameters.
2. To assess to what extent the amount of Ca and P within an unbanded egg component (shell, shell membrane, yolk or albumen) is affected by the clutch size, the date of laying within a laying season, the female's pond of residence, the total wet mass of the component and the concentration of the element within that component.

4.4 Materials and methods

All procedures were approved by the University of Pretoria Animal Ethics Committee (certificates v104-16 and v109-15 (Appendix F)).

4.4.1 Specimen collection and preparation

Unbanded *C. niloticus* eggs were collected from two incubators on a single commercial farm, once viable eggs within the incubation box had hatched.

Eggs were cleaned, opened and their components separated and weighed as detailed previously by Brown *et al.* (2019). Only eggs with a clear, uncontaminated albumen (n = 185) were considered for further processing and analysis. Combined shell and shell membranes were oven dried at 50 °C for six to eight hours (Labcon, Ferndale, South Africa), after which the two were manually separated and their respective dried masses determined.

Direct measurement of the dry mass fraction of unbanded egg shells could not be performed, because clean separation of wet shell and shell membrane of unbanded eggs was not possible. For the research study described in Chapter 3, as well as later in Chapter 5 of this thesis, fertile egg shell and shell membrane were separated while wet, and the dry fraction of fertile eggshell was determined. Since unbanded and fertile shell and shell membranes were macroscopically highly similar in appearance and texture, for this study an assumption was made that the dry

fraction of unbanded egg shell and shell membranes was identical to that measured for fertile eggs. A mean dried mass fraction of 30 fertile eggshells was used as a proxy for the dry mass fraction of unbanded shells. An estimate of the wet mass of unbanded eggshells and shell membranes was thus determined from their respective estimated dried mass fraction.

A weighed aliquot of each yolk and albumen sample was freeze-dried at −50 °C, 80 mTorr (Air and Vacuum Technologies, Midrand, South Africa) and oven-dried at 50 °C to constant mass. The dried aliquot was then weighed, and the dried mass proportion of yolk and albumen determined.

4.4.2 Sample analysis

Analysis was performed by a commercial laboratory under supervision. A pilot analysis of 24 eggs was performed to check efficacy of sample digestion and identify potential sources of measurement error.

4.4.3 Sample digestion and analysis

Approximately 0.2 g of each sample was weighed into clean Teflon microwave digestion tubes using a laboratory microbalance (Micro Precision Precisa XT220A (Appendix E)). Ten millilitres of 70% HNO_3 (Sigma-Aldrich, Johannesburg, South Africa) was added to each digestion tube using a 10 mL micropipette (Eppendorf, Dubai, UAE). Samples were pre-digested for 15 min while swirling at room temperature, then 1 mL of 60% H_2O_2 (Sigma-Aldrich, Johannesburg, South Africa) was added before digesting for a further 15 min at room temperature.

Digestion tubes were placed in a microwave digester (Mars 6, CEM Microwave Technology Limited, Buckingham, UK (Appendix D)) and digested for 50 min (ramp-up time 20 min to 200 °C, hold time 30 min). After digestion and cooling to room temperature, the sample was rinsed from the digestion vessel into an empty 50 mL pre-weighed polypropylene tube

(Plastpro, Johannesburg, South Africa). The net mass of liquid solution was determined by subtraction. One millilitre of this solution was aspirated with a Rainin variable micropipette (Mettler Toledo, Greifensee, Switzerland) and weighed in grams, accurate to two decimals.

The density of the liquid sample could then be determined using the formula density = mass / volume, and the total volume of the solution could be determined using the formula:

Volume = ((Combined mass of container and sample) – (Container mass))/ (density of liquid)

An Agilent 5100 (Agilent Technologies, Santa Clara, California, USA) inductively coupled plasma, optical emission spectrometer (ICP-OES) equipped with an autosampler was used for sample analysis. Argon plasma flow rate was 15 litres per minute and nitrogen shear gas flow rate was 1.2 litres per minute. Nebuliser (Agilent OneNeb Series 2, Agilent Technologies, Santa Clara, California) sample flow rate was 0.6 litres per minute. Wavelengths of 370.602 nm (Ca) and 178.222 nm (P), both measured radially, gave the most consistent measurements. Sample concentration was determined from the mean of three replicates.

Calcium and phosphorus standards (Inorganic Ventures, Christiansburg, Virginia, USA) diluted to concentrations of 1, 10, 20, 50, 100, 250 and 400 mg/L were used. A 1% HNO_3 solution was used as a sample blank. A calibration curve with an R-squared value of 0.9999 was considered acceptable. Limit of detection (LOD) and limit of quantification (LOQ) for each element were determined by calculating the standard error of the emission value for each concentration value on the ICP-OES Ca and P standard emission curve, and then dividing this figure by the slope of the regression line. The LOD was calculated as 3 times this value, and the LOQ as 10 times this value (Shrivastava, 2011).

4.4.4 Control of quality and accuracy of analysis

A powdered multivitamin certified reference material (CRM) was used to evaluate potential matrix effects and provide a measure of method and instrument accuracy (Standard Reference Material 3280, National Institute of Standards and Technology (NIST), Gaithersburg, Maryland (Appendix A)). Certified concentrations for Ca were 110.70 g/kg with an allowable variation of 5.30 g/kg above or below this value, and for P, 75.70 g/kg with an allowable variation of 3.20 g/kg. Twenty-six CRM sub-samples were separately weighed, digested and analysed. A blank sample, a multivitamin NIST CRM sample, as well as samples of acid-dissolved pure KH_2PO_4 equivalent to 227.59 g/kg P (Appendix C) and pure $CaCO_3$ equivalent to 400.44 g/kg Ca (Appendix B) were analysed after every 20 samples to check for instrument drift.

Intra-assay variability in sample analysis was determined by including blind duplicate samples of shell (n = 11), shell membrane (n = 11), yolk (n = 8) and albumen (n = 6) among submitted samples.

4.4.5 Specimen Ca and P determination

Of 185 eggs with an albumen clarity score of one, samples from 95 unbanded eggs from 78 clutches were analysed. All 95 had Ca and P concentration measured for shell membrane, yolk and albumen. A pilot analysis showed low variability in shell Ca concentration and negligible shell P concentration, so to save costs, only 29 shell samples (from 15 clutches) were analysed.

4.4.6 Data analysis

Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used for data collation and Stata 14 (Statacorp, College Station, Texas, USA) for statistical analysis.

4.4.6.1 Assessment of measurement accuracy

The mean, SD and CV were determined for each set of reference material results. These values were then compared with certified values to gauge measurement accuracy.

The intra-assay mean, SD and CV were determined for each duplicate sample pair.

4.4.6.2 Concentration and total content of Ca and P in unbanded egg components, and the grouping influence of clutch on these measurements.

After assessing normality of distribution, outliers greater than three standard deviations from the mean were noted and excluded. For normally distributed variables, the mean and standard deviation were determined for the concentration and content of Ca and P in each component. Where the distribution was found to be non-normal, the median, 25^{th} and 75^{th} percentiles were used. The proportional contribution of each component to total egg Ca and P was described.

The intracluster correlation coefficient was determined for each component's Ca and P concentration and content to assess the grouping effect of clutch on these variables.

4.4.6.3 Assessment of the effect of covariates on concentration and content of Ca and P in each component.

To eliminate possible clutch effects as a source of bias, a single egg was randomly selected from each of the 78 clutches. Ca and P concentration and content of each component were graphed using histograms and visually inspected for normality of distribution. The Stata command "sktest" was used to formally assess normality of distribution at the 5% significance level. In certain cases, extreme outlying data points (greater than 3 SD from the mean) were excluded. If, after excluding these outliers, the histogram and sktest suggested that the concentration or content of Ca or P in a component was still non-normally distributed, a log (base 10) transformation was performed. Multiple regression was then used to assess the effect of the potential predictor variables clutch size, lay date, pond of origin and component mass on

the outcome variables of Ca and P concentration per component. Non-significant variables ($P > 0.05$) were successively excluded in sequence of decreasing P-value. Using the same predictor variables (with the addition of Ca and P concentration), the multiple regression was repeated for the outcome variables of Ca and P content per component. All regression models were checked for normality and heteroscedasticity of residuals (Breusch-Pagan / Cook-Weisberg test).

For eggshell Ca concentration and content, a low number of shell samples were analysed (29 shells from 15 clutches). A trial analysis with a multi-level mixed-effect model was used, with clutch as a stochastic second-level grouping variable. A likelihood ratio test was used to compare this model to a multiple regression model without considering the effect of clutch. If the likelihood ratio test was not significant ($P > 0.05$), a multiple regression model, without clutch as grouping variable, was used.

4.5 Results

4.5.1 Assessment of measurement accuracy

For Ca, the LOD was 0.01 g/kg and the LOQ was 0.04 g/kg. For P, the LOD was 0.002 g/kg and the LOQ was 0.007 g/kg. Mean CRM Ca measured 2.70 g/kg (2.6%) lower than the lower limit of the certified concentration, and 7.95 g/kg (7.2%) lower than the mean certified concentration (measured mean = 102.75 g/kg, SD = 1.26, n = 26). Mean CRM P measured 0.30 g/kg (0.4%) higher than the lower limit of the certified concentration and 2.90 g/kg (3.8%) lower than the mean certified concentration (measured mean = 72.82, SD = 1.00, n = 26). Calcium carbonate equivalent to a known 400.44 g/kg Ca was measured at 1.4% lower than expected (measured mean = 395.03, SD = 2.74, n = 22), while monopotassium phosphate equivalent to 227.59 g/kg P was measured at 4.4% lower than expected (measured mean = 217.57, SD = 3.74, n = 22).

Duplicate yolk and eggshell samples showed the lowest variability in concentrations. P was below the limit of detection for all shell samples in the present study (Table 4.1). Shell membrane Ca concentration was highly variable among duplicate samples.

Table 4.1.
Summary of average intra-assay CV (%) of duplicate Ca and P concentration measurements

	Ca	P	n
Shell	0.34	—[a]	11 pairs
Shell membrane	15.54	4.15[b]	11 pairs
Albumen	1.47	2.76	6 pairs
Yolk	0.54	0.52	8 pairs

a. P was below the limit of detection for all shell samples
b. Shell membrane P was not reliably detected in three samples submitted for determination of intra-assay CV.

4.5.2 Relationship between element content, element concentration and component mass

Figure 4.1 summarizes the association between the amount of Ca or P in a component, the concentration of the element in that component, and the mass of the component. It is evident that wet mass of the shell and yolk has by far the greatest effect on the content of shell Ca (Figure 4.1b) and yolk Ca (Figure 4.1l) and P (Figure 4.1n) respectively. Although in shell membranes, the concentration and content of Ca as well as those of P are linearly related, the amount of each element is tiny. The amounts of Ca in the shell, and those of Ca and P in the yolk greatly exceed that found in the albumen and shell membrane.

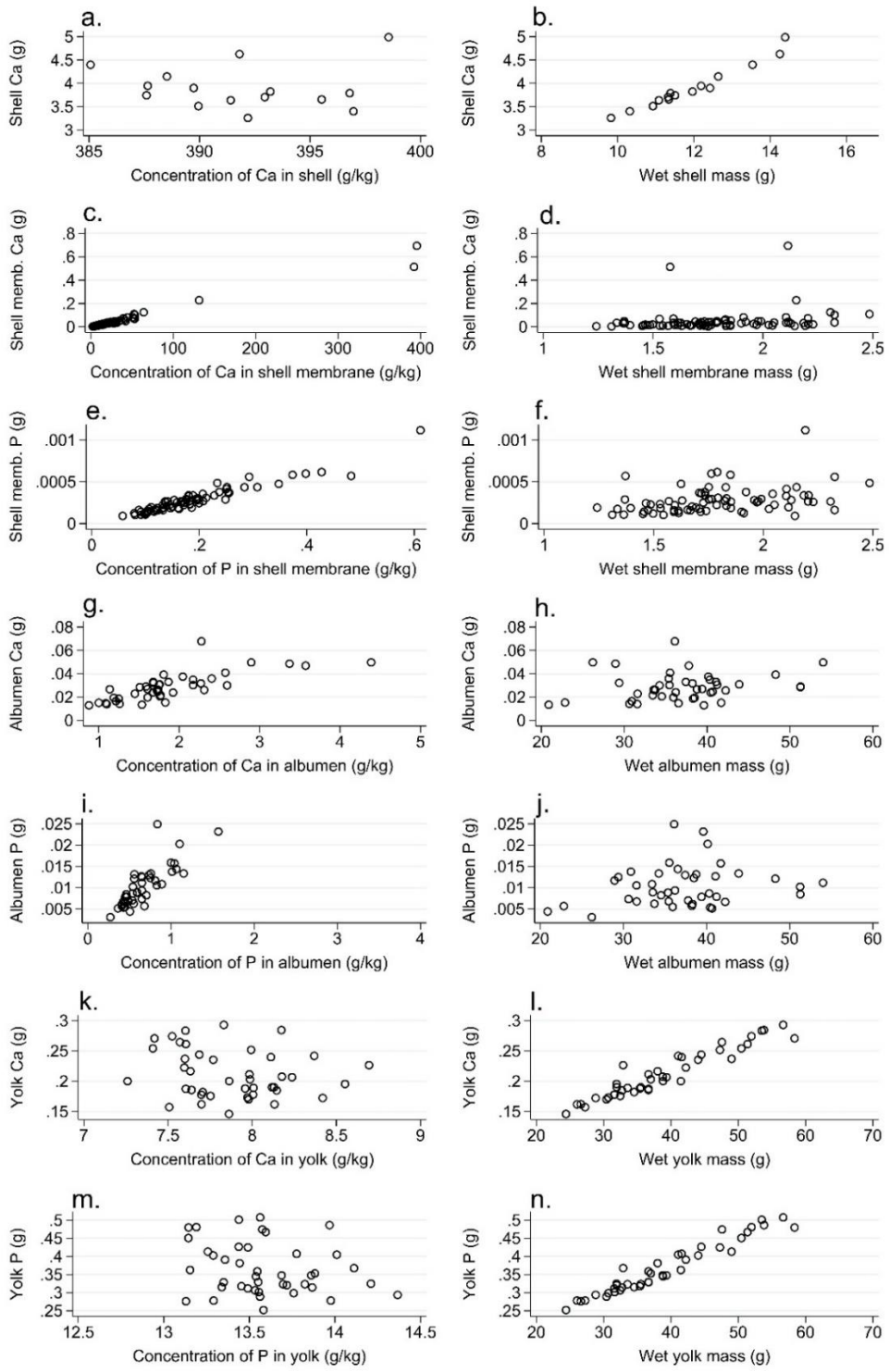


Figure 4.1.
Association between egg component Ca or P content, and the concentration of each element in that component (graphs a, c, e, g, i, k and m), or the wet mass of the component (graphs b, d, f, h, j, l, and n).

4.5.3 Concentration and total content of Ca and P in each unbanded egg component

Descriptive statistics for the concentrations of Ca and P are presented in Table 4.2, and those for the content of Ca and P in each component are given in Table 4.3. For normally distributed variables the mean and standard deviation is given, while for non-normally distributed variables, the median, 25th and 75th percentiles are provided.

Table 4.2.
Concentrations of Ca and P in each egg component (g/kg); (one randomly selected egg per clutch)

	Mean	SD	Median	p25[a]	p75[b]	n
Ca						
Shell	391.87	3.90	—	—	—	15
Shell membrane	—	—	17.88	9.98	28.82	74[c]
Albumen	—	—	1.72	1.33	2.17	78
Yolk	7.83	0.34	—	—	—	78
P						
Shell membrane	—	—	0.17	0.12	0.21	75
Albumen	—	—	0.55	0.45	0.75	78
Yolk	13.56	0.34	—	—	—	78

a. 25th percentile
b. 75th percentile
c. Three outliers evident in Figure 4.1c and d were excluded

Table 4.3.
Ca and P content (in grams) of each egg component (one randomly selected egg per clutch)

	Mean	SD	Median	p25[a]	p75[b]	n
Ca						
Whole egg	4.22	0.65				6
Shell	3.90	0.47	—	—	—	15
Shell membrane	—	—	0.03	0.01	0.04	74
Albumen	—	—	0.027	0.02	0.033	42
Yolk	0.21	0.04	—	—	—	42
P						
Whole egg	0.38	0.07	—	—	—	42
Shell membrane	—	—	0.00026	0.00016	0.00034	75
Albumen	—	—	0.009	0.007	0.013	42
Yolk	0.37	0.07	—	—	—	42

a. 25th percentile
b. 75th percentile

The relative contributions of each component to the total Ca or P content of the egg are shown in Table 4.4.

Table 4.4.
Percentage contribution of each component to egg Ca and P content (one randomly selected egg per clutch)

	Mean	SD	Median	p25[a]	p75[b]	n
Ca						
Shell	91.77	3.79	—	—	—	15
Shell membrane	—	—	1.52	0.48	1.95	15
Albumen	—	—	0.70	0.57	0.89	15
Yolk	4.67	0.54	—	—	—	15
P						
Shell membrane	—	—	0.07	0.05	0.09	42
Albumen	—	—	2.45	1.98	3.69	42
Yolk	97.08	1.46	—	—	—	42

a. 25th percentile
b. 75th percentile

The intracluster correlation coefficients (r_{ic}) for Ca and P concentration and content are summarised in Table 4.5. Three high outliers were excluded from the determination of the r_{ic} for shell membrane Ca concentration and content, because they likely represented contamination by the shell. Clutch had a weak grouping effect on shell Ca concentration, but a strong grouping effect on shell Ca content. Clutch had a slightly stronger grouping effect on yolk Ca and P concentration than it had on yolk Ca and P content. Clutch had a very weak grouping effect on albumen Ca and P concentration as well as content (refer to Table 4.5).

Table 4.5.
Intracluster correlation coefficients (ric) for the concentration and content of Ca and P in each egg component

	r_{ic}	n eggs	n clutches
Ca concentration			
Shell	0.39	29	15
Shell membrane	0.63	28	14
Albumen	0.12	32	15
Yolk	0.79	32	15
P concentration			
Shell membrane	0.59	29	15
Albumen	0.17	32	15
Yolk	0.76	32	15
Ca content			
Shell	0.87	29	15
Shell membrane	0.75	28	14
Albumen	0.20	14	8
Yolk	0.64	16	9
P content			
Shell membrane	0.69	29	15
Albumen	0.15	16	9
Yolk	0.68	16	9

4.5.4 Assessment of the effect of covariates on content of Ca and P in each component.

Of the potential predictor variables clutch size, date of laying, female's pond of residence, wet mass of the component and component element concentration, the covariates that most influenced the content of Ca and P in each component were element concentration and component mass.

In the regression models, albumen and yolk Ca and P content and concentration appeared in some cases to be statistically influenced by pond, date of laying and clutch size. However, relatively small numbers of specimens in each group together with very small coefficients (10^{-3} to 10^{-4}), makes meaningful biological interpretation dubious. For example, yolks of eggs from Pond 3 had an apparently lower Ca concentration than those of Pond 0 (coefficient: –0.32 g/kg, P = 0.03), however Pond 3 contributed only seven eggs to this model, while Pond 0, which contributed the greatest number, contributed 36.

4.5.4.1 Shell Ca content

A very small variation in shell Ca concentration was found (refer to Table 4.1, Figure 4.1a), and a likelihood ratio test showed no advantage to using a multilevel model over a simple regression model for shell Ca content measurements (P = 0.055). Shell Ca content was normally distributed. The regression model was a good fit (F statistic (7, 21 df) = 51.55, P = 0.001, $r^2 = 0.95$, n = 29). Wet shell mass was the only significant predictor of shell Ca content: for each gram that wet shell mass increased, the shell Ca content increased by 0.27 grams (SE = 0.02, 95% CI = 0.23–0.32, df = 21, $t = 12.25$, P < 0.001).

4.5.4.2 Shell membrane Ca and P content

Despite removing gross outliers greater than three standard deviations from the mean, shell membrane Ca content was not normally distributed and required log transformation. The model

for predicting log transformed shell membrane Ca content was a good fit (F statistic (8, 63 df) = 6859.09, P < 0.001, r^2 = 0.99, n = 72).

The log of shell membrane Ca content is estimated as −3.26 + 0.23 (wet shell membrane mass in grams) + 1.00 (log of shell membrane Ca concentration in g/kg)

(SE of coefficient of wet shell membrane mass = 0.006, 95% CI = 0.22–0.25, *t* = 41.43, P < 0.001; SE of coefficient of log of shell membrane Ca concentration= 0.005, 95% CI = 0.99–1.01, *t* = 209.76, P < 0.001).

After removing an outlier, the model for predicting log transformed shell membrane P content was a good fit (F statistic (8,65 df) = 3098.51, P < 0.000, r^2 = 0.99, n = 74).

The log of shell membrane P content is estimated as −3.24 + 0.23 (wet shell membrane mass in grams) + 1.02 (log of shell membrane P concentration in g/kg)

(SE of coefficient of wet shell mass = 0.005, 95% CI = 0.22–0.24, *t* = 44.21, P < 0.001; SE of coefficient of log of shell membrane P concentration = 0.01, 95% CI = 1.00–1.03, *t* = 137.24, P < 0.001).

4.5.4.3 Albumen Ca and P content

The model for predicting albumen Ca content was a good fit (F statistic (7, 31 df) = 27.23, P < 0.001, r^2 = 0.86, n = 39). If albumen Ca concentration increased by 1 g/kg, albumen Ca content increased by 0.01 grams (SE = 0.001, 95% CI = 0.01–0.02, df = 31, *t* = 11.82, P < 0.001). Wet albumen mass (in grams) was also predictive of albumen Ca content: for each increase by one gram in albumen mass, albumen Ca content increased by 0.006 g (SE = 0.001, 95% CI = 0.007–0.016, df = 31, *t* = 5.44, P < 0.001).

The model for predicting albumen P content was a good fit (F statistic (2, 36 df) = 108.26, P < 0.001, r^2 = 0.86, n = 39). Albumen P concentration had a significant effect on albumen P content (coefficient 0.014, SE = 0.001, 95% CI = 0.012–0.017, *t* = 14.20, P < 0.001). The wet

mass of albumen (in grams) had a tiny but statistically significant effect on total albumen P (coefficient 0.0002, SE = 0.00004, 95% CI = 0.0001–0.0002, t = 4.53, P < 0.001).

4.5.4.4 Yolk Ca and P content

Controlling for other covariates, the Ca content of the egg yolk increased by a mean of 0.005 g for each increase by one gram in wet yolk mass (SE = 0.0003, 95% CI = 0.004–0.005, df = 32, t = 18.6, P < 0.001), and increased by 0.03 g for each increase by one g/kg in the yolk Ca concentration (SE = 0.005, 95% CI = 0.02–0.04, df = 32, t = 6.15, P < 0.001).

The P content of egg yolk increased by a mean of 0.03 g for each increase by one g/kg in the concentration of P in the yolk (SE = 0.009, 95 % CI = 0.012–0.048, df = 32, t = 3.43, P < 0.05).

Controlling for other covariates, the P content of egg yolk increased by 0.008 g for each increase by one gram in the wet mass of the yolk (SE = 0.0005, 95 % CI = 0.007–0.009, df = 32, t = 18.32, P < 0.001).

4.6 Discussion

Reference material Ca and P concentrations measured consistently lower than certified values, by between 1.4 and 7.2%. This systematic error may have been caused by issues in sample processing and analysis, including incomplete sample digestion, incomplete sample nebulisation, or chemical matrix effects (Gaines, 2011).

Given this error, it is logical to assume that the actual concentration of Ca and P in egg samples was also slightly higher than measured. However, the error was present across all sample types, so while absolute values may be under-reported, the relative values are useful in comparing Ca and P in different egg components.

Duplicate submissions of eggshell and yolk specimens yielded consistent concentration measurements (mean CV less than 0.6%), which suggests that laboratory sample handling and analysis was consistent, and that yolk and eggshell samples were by nature homogeneous. By contrast, duplicate shell membrane samples yielded highly variable concentrations from successive analyses. It is suspected that the flaky, highly heterogenous shell membrane samples may have been contaminated by shell during separation of these two components. This was particularly evident in shell membrane Ca concentration, which had extremely large mean CV.

Albumen Ca and P concentrations both had high intra-assay coefficients of variation compared to those of shell and yolk. Freeze-dried albumen was a delicate, macroscopically homogeneous white powder. Without further investigation it is difficult to explain the relatively large intra-assay CV for this component, however subtle contamination of some albumen samples by yolk cannot be ruled out. Such contamination may have occurred either during sample processing, or during the prolonged interval between egg laying and hatching.

Regression models for Ca and P concentration failed to explain much of their variability. Besides variation in Ca and P concentration of shell membrane and albumen specimens due to technical issues already described, there may be unmeasured causes of variation in Ca and P concentration seated in females and ponds. An attempt was made to restrict the effect of female in regression models by selecting just one egg from each clutch. In the case of shell Ca concentration, where two eggs from each of 15 clutches were used, it was confirmed beforehand that clutch (or female) only had a weak grouping effect. Despite this, females differed with respect to unmeasured variables, such as age, parity and geographic origin. No attempt was made to balance the data with respect to these variables. Eggs used for the regression analyses came from five different breeding ponds. Although pond was included as a factor in all regression models, two ponds contributed only seven eggs each, one pond contributed 15 eggs, a fourth contributed 20, and the fifth contributed 46. This low per-pond

sample contribution reduced the ability to accurately assign biological significance to statistically significant yet marginal differences between ponds. A further complication is that unmeasured variables that differ from female to female are confounded with those seated in ponds, since each female lived in one pond only.

The most important factors influencing the Ca and P content of unbanded egg components, were element concentration and component mass. Other factors such as pond and date of laying within laying season were statistically significant in some models, but typically had very low coefficients and thus minimal impact on their respective regression models.

As might be expected, the shell is the source of the majority (about 92%) of the Ca in the egg. The highly similar concentrations of shell Ca among samples from different clutches laid by different females implies that the shell gland of any female Nile crocodile consistently produces a calcium carbonate shell of uniform Ca concentration. This low variability, together with a low intracluster correlation coefficient for shell Ca concentration suggests that a small number of randomly selected eggs, with no regard to their clutch of origin, is a fair sampling strategy to determine the mean Ca concentration of eggshells in a population.

In the multivariable regression model, shell mass was the only significant predictor of shell Ca content: shell Ca concentration was non-significant, underpinning the finding that Ca concentration in the eggshell has very low variability. Bigger eggs have more shell Ca for no other reason than that they have more shell. As was noted for *Crocodylus porosus* (Burley *et al.*, 1987), *C. niloticus* eggs tend to be of similar size within clutches (Brown *et al.*, 2019): the high intracluster correlation coefficient for shell Ca content showed that their shell Ca content is also very similar within clutches relative to between clutches.

Since eggshell has a similar Ca concentration as pure calcium carbonate, if analytical equipment is unavailable one may gain a fair estimate of the Ca content of the unbanded

eggshell simply by multiplying the concentration of Ca in dry calcium carbonate (about 40%, or 400 g/kg) by the dried mass of the eggshell.

Given the large variability in shell membrane Ca among the samples analysed, and the apparent within-sample heterogeneity shown using duplicate measurements of Ca concentration, all results for shell membrane Ca in the present study should be interpreted with caution. Compared to shell Ca and yolk Ca and P concentrations, shell membrane P concentration showed a large range in values. In an unbanded egg, it would be expected for shell membrane to be an inert lining of the shell with a consistent P content. The large variation found in the present study is likely indicative of a processing issue. An alternative cause of such variability in concentration could be the unpredictable exchange of Ca and P with other components in the incubator prior to egg collection. However, given the size of the variability, it seems that gross contamination of some samples is a more likely cause.

Mean concentration and content of Ca and P in the albumen was very low, and varied comparatively widely. Both albumen Ca concentration and wet mass of albumen were predictive of albumen Ca content, however the concentration of Ca in the albumen had a far greater effect than wet albumen mass. The albumen contained on average less than one percent of total egg Ca and three percent of albumen P, so it seems unlikely that it is a significant contributor to the developing foetus' requirements of these elements.

Yolk had the highest concentration of P of all unbanded egg components, with very low variability among sampled eggs. Yolk also had a relatively high concentration of Ca. The yolk contributed by far the largest amount of P and the second largest amount of Ca to the egg. This finding suggests that the yolk is the primary source of P for the developing foetus, which is not surprising given that it contains substantial quantities of vitellogenin and other phosphate-rich lipoproteins (Noble *et al.*, 1991).

The present research enhances knowledge of the distribution of Ca and P in the components of the unbanded crocodilian egg. It will facilitate the study of Ca and P metabolism in the fertile egg of the Nile crocodile, which will expand on work done by Packard and Packard (1989). Ca is present in substantial quantities in the shell and yolk, and P in the yolk. These components represent the primary sources of these elements for the developing foetus and should be the focus of samples for future research into preventive or therapeutic interventions, particularly if the cost of chemical analysis restricts sample numbers.

4.7 Acknowledgements

The researchers would like to thank Mr Stefan van As and Ms. Karon Griffiths for provision of sample material and assistance during data collection, as well as Mr Kgomotso Sekwele for assistance in egg processing.

4.8 Sources of funding

Funding for this research was provided by University of Pretoria in the form of a bursary to the first author, and by the South African National Research Foundation (NRF) in the form of an incentive fund for rated researchers to the last author (grant number 85836).

4.9 Declaration of conflicts of interest

None

Chapter 5. Calcium and phosphorus in fertile eggs of the Nile crocodile (*Crocodylus niloticus*)

The contents of this chapter will be submitted in modified form to a peer-reviewed journal.

5.1 Abstract

The source of calcium (Ca) and phosphorus (P) for the developing Nile crocodile (*Crocodylus niloticus*) foetus has not been studied, however research in other species suggests that it is principally derived from yolk and shell. Using inductively coupled plasma optical emission spectroscopy (ICP-OES), the Ca and P concentration and content of 30 fertile eggs was determined within 10 days of hatching, and findings were compared with those for size-matched unbanded eggs (eggs which fail to form an opaque band around the lesser circumference, indicative of failure of fertilisation or early embryonic development). For Ca, shell contained the highest concentration and content, followed by the foetus and then the intra-abdominal yolk. For P, the foetus contained the highest concentration and content, followed by the intra-abdominal yolk. Yolk Ca and P concentration did not vary widely among unbanded eggs, but was quite variable in the intra-abdominal yolk of late-incubation foetuses within fertile eggs. Yolk Ca concentration of fertile eggs was in some cases found to exceed that of unbanded eggs, suggesting that Ca is stored there after being removed from the shell, however Ca content (defined as concentration multiplied by mass) was consistently lower among fertile eggs, indicating net yolk Ca depletion. Yolk P concentration and content of fertile eggs was consistently lower than that of unbanded eggs, suggesting a net depletion of yolk P reserves, without replenishment.

5.2 Keywords

Calcium, phosphorus, embryo, egg, crocodile, crocodilian, alligator, egg.

5.3 Introduction

Calcium (Ca) is essential for normal physiological function in animals, including mineralisation of bone and teeth, where it complexes with phosphate ions in the form of

hydroxyapatite (Carafoli, 1987). It also functions in nerve conduction, intracellular signalling, blood coagulation and as an enzyme cofactor. Phosphorus (P) is required for bone formation, incorporation into cell membrane phosphoproteins, and is an integral constituent of nucleic acids (Shaker and Deftos, 2018). It functions in intracellular fluid balance, and is an essential component of adenosine triphosphate (ATP) (Thompson *et al.*, 2013). Abnormalities of Ca and P metabolism can result in a variety of skeletal disorders among captive reptiles (Marcus, 1981). In crocodilians these disorders may include glassy teeth, rubbery jaws and an increased incidence of fractures (Huchzermeyer, 2003, Lane *et al.*, 1984, Manolis and Webb, 2016).

The role of abnormal Ca or P metabolism *in ovo* on the subsequent performance of the hatchling in a farmed situation has not been formally studied. Shilton *et al.* (2014) showed that mild hypocalcaemia and moderate hypophosphataemia, as well as distinct hypoalbuminaemia and osteoporosis occurred more commonly in runt *Crocodylus porosus* hatchlings than in normal sized hatchlings. The authors speculated that this may have been associated with post-hatching inanition.

The contributions of the various components of the fertile egg to Ca and P in the foetus have been studied in *Alligator mississippiensis* (Packard and Packard, 1989) and in *Crocodylus novaeguineae* (Jenkins, 1975). Ca and P in the Nile crocodile egg have not been investigated.

During embryonic development in oviparous species, Ca from extra-embryonic sources (yolk or shell) is moved across the CAM and yolk sac membrane to the conceptus (Packard and Clark, 1996). In lizards and snakes, most Ca comes from the yolk. By successively opening eggs through the incubation period and analysing the Ca and P content of its various components, Packard and Packard (1989) showed that the shell of *A. mississippiensis* was the main source of foetal Ca, with yolk serving as a temporary storage for shell-derived Ca in the last third of incubation.

In addition to the Nile crocodile's utility to man as an aquaculture species in the production of leather and meat, it is also a long-lived apex predator in African freshwater ecosystems (Pooley, 1982), and is thus likely to be a useful sentinel species in the detection and quantification of cumulative environmental toxins (Lundholm, 1997). In birds, organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) have long since shown to alter eggshell calcification (Ratcliffe, 1967, Hickey and Anderson, 1968). Research in other crocodilian species has shown that normal eggshell anatomy and physiology may be altered by exposure to environmental pollutants (Stoker *et al.*, 2013). Given that organochlorines may affect the Ca status of bird and crocodilian eggs, description of the concentration and content of Ca in egg components may be of value under conditions where organochlorine toxicity is suspected.

Before studying the effect of dietary supplements or alterations in management protocols on egg or hatchling Ca or P, methods must be developed to separate, process and analyse samples to yield accurate, representative results. The natural variability in concentrations and amounts of Ca and P in each component must be described for normal eggs, and potential confounding factors that could affect these measurements must be identified. The principle sources of Ca and P for incorporation in the foetus must be identified, together with an approximation of each component's relative contribution. This will allow for comparative study of pathologies of Ca and P metabolism in Nile crocodile eggs in captive and wild populations.

In the fertile crocodile egg, development of the chorioallantois in early incubation results in physical changes to the shell membrane that are visible through the shell. This process is known as 'banding': eggs in which this fails to occur (due either to lack of fertilisation or early death of the embryo) are known as 'unbanded' eggs. In the research presented in this thesis, freely available unbanded eggs were used as proxies for very early-stage fertile eggs, under the assumption that unbanded eggs reflect the Ca and P content and distribution of fertile eggs prior to the onset of embryonic metabolic activity.

Chapter 4 of this thesis described the variation in Ca and P concentration and content of unbanded eggs, and investigated the effect of potential confounding variables on these parameters. The present chapter focused on the fertile egg, and had the following aims:

1. To describe the concentration and content of Ca and P in the components (shell, shell membrane, yolk, foetal membranes and foetal fluids, and foetus) of fertile eggs.
2. To assess the relationship between foetal Ca and P content and that of the other fertile egg components.
3. To determine the main sources of Ca and P for the developing conceptus by comparing fertile eggs to unbanded eggs of similar mass.

5.4 Materials and methods

All research procedures were approved by the University of Pretoria Animal Ethics Committee (approval certificates v104-16 and v109-15 (Appendix F)). All eggs used in this study were collected in 2016 from a commercial crocodile farm in the North West Province, South Africa.

5.4.1 Specimen collection and preparation

Unless stated otherwise, all laboratory equipment and methods (such as freeze- or oven-drying) used in preparation of fertile egg samples was identical to that used for unbanded eggs. All fertile egg specimens were digested and analysed in batches with unbanded egg samples. For a more detailed description of egg processing, refer to Chapter 2 of this thesis. Killing of foetuses within fertile eggs was performed according to IUCN-CSG recommended practices (Manolis and Webb, 2016).

Briefly, fertile *C. niloticus* eggs (n = 30; one from each of 30 clutches) were carefully collected between ten and five days prior to their predicted hatching date, uniquely numbered and placed immediately on ice. All eggs originated from the same farm, but from different breeding ponds during the same season. At the processing laboratory, eggs were refrigerated for 24 h at 4 °C

before each egg was cleaned with a swab and deionised water and allowed to dry for at least an hour at room temperature. The mass of each egg was determined in grams, to three decimals. The shell overlying one pole of the egg was broken, and shell fragments removed and kept for later weighing, resulting in a shell-free region about 30 mm in diameter. The shell membrane was cut with a scalpel and the foetus removed from the egg with rat-toothed forceps. The attachment between the intra-abdominal yolk sac and the allantochorion was cut with scissors.

To ensure the foetus was dead and not in a cold-induced torpor, it was decapitated prior to dissection. No haemorrhage nor movement occurred during decapitation. The intra-abdominal yolk within its perivitelline membrane was removed from the abdomen. Remaining foetal fluids, remnant albumen and foetal membranes were removed from the empty egg and placed in a weighing container. The shell was separated from the shell membrane. All components of the fertile egg (shell, shell membrane, yolk, foetus, and a mixture of foetal membranes, foetal fluids and remnant albumen) were weighed separately in pre-weighed containers and their masses determined by subtraction. The foetus was homogenised. An aliquot of homogenised foetal tissue, the entire yolk, and the entire mixture of foetal fluid, foetal membrane and remnant albumen were stored in separate containers for freeze drying. After freeze drying, samples were oven dried to constant mass. The shell and shell membrane were oven dried for at least six hours at 50 °C.

5.4.2 Specimen Ca and P determination

Ca and P concentrations were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES) for each component of 30 fertile eggs (shell, shell membrane, yolk, foetus, and a mixture of foetal membranes and residual foetal fluids). Details of the process of analysis and the determination of limits of detection and quantification are identical to those for unbanded eggs, and are provided in 4.4.3.

Ca and P content was calculated by multiplying dry component mass by element concentration.

5.4.3 Assessment of measurement accuracy

Methods used to assess accuracy of measurement using certified reference materials and quality control samples are identical to those used for unbanded eggs and are reported in 4.4.4. As was done for unbanded eggs, the within-sample method repeatability and specimen homogeneity was assessed by splitting the specimens derived from six eggs (shell, shell membrane, yolk, foetus and a mixture of foetal membrane and foetal fluid) into paired aliquots and submitting each of these to the laboratory as separate samples.

5.4.4 Matching of unbanded and fertile eggs

To compare unbanded eggs with similar fertile eggs, each fertile egg was matched to an unbanded egg of which the mass was as close as possible to, but within five grams above or below its own mass. When selecting pairs, the only criterion considered was egg mass: other potential covariates such as laying date, pond of origin on the farm and clutch of origin were not considered.

For unbanded eggs, separation of shell membrane from shell was performed on dried samples. As discussed in 4.4.1, the wet mass of unbanded shell and shell membrane was estimated by assuming that they respectively contained the same fraction of moisture as that of fertile eggs, for which both wet and dry masses were measured.

For shell membrane and yolk, pairing fertile eggs with size-matched unbanded eggs allowed for a direct comparison of measured Ca and P concentration and content. However, only three of 30 unbanded shell samples used in the prior study (Chapter 4) came from eggs that could be size-matched to fertile eggs. Cost constraints prevented the analysis of more samples. Therefore, to compare shell Ca content of unbanded eggs with size-matched fertile eggs, a prior finding was exploited: namely that the unbanded eggshell Ca concentration was almost the

same among a random selection of eggs (refer to Table 4.2). The mean shell Ca concentration of 29 analysed unbanded eggs was 391.21 g/kg (SD 3.78 g/kg, min 384.22 g/kg, max 398.56 g/kg). This equated to a very low CV of 0.97%, indicating that the mean value for Ca concentration was a good approximation of the Ca concentration in all unbanded eggshells. The principle determinant of Ca content in an eggshell was the mass of that eggshell (refer to Figure 4.1 a and b). The dry shell mass was therefore multiplied by the mean concentration value (391.21 g/kg) to provide an estimate of the shell Ca content of a given egg.

5.4.5 Data analysis

Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) was used for collation of data, and Stata 14 (Statacorp, College Station, Texas, USA) was used for statistical analysis.

5.4.5.1 Assessing normality of distribution

Ca and P concentration and content for each component were graphed using histograms and visually inspected for normality of distribution. The Stata command "sktest" was used to formally assess normality of distribution at the 5% significance level.

5.4.5.2 Descriptive statistics

For normally distributed concentration and content variables, the mean, standard deviation, minimum and maximum were reported. For non-normally distributed concentration and content variables, the median, 25th and 75th percentiles, minimum and maximum were reported.

5.4.5.3 Comparison of Ca or P concentration and content between size-matched pairs

Ca and P concentration were determined on a dry matter basis. Unfortunately, data for the total dry mass of 14 of the 30 size-matched unbanded yolk and albumen samples was missing. This meant that only 16 eggs could have their yolk and albumen Ca and P content determined. Comparison was confined to these 16 pairs.

A primary aim of the study was to determine whether fertile eggs differed from size-matched unbanded eggs in their component Ca and P concentration and content. Egg mass was the only factor considered when selecting an unbanded egg to pair with each fertile egg: other potential confounding variables were not considered. By the time the 30 fertile eggs were made available, the collection of unbanded eggs was already complete and significant effort had been put into opening, separating, sorting and drying their components. To optimise measurement accuracy, unbanded eggs to form sample pairs had to be chosen from those with albumen clarity score of 1 (refer to Figure 2.11), which greatly reduced the potential pool of samples. The imposition of further restrictions (such as ensuring that paired eggs were selected from the same pond or clutch) would have made the selection of an adequate number of pairs impossible.

In performing the statistical analysis, it was therefore necessary to use a statistical model that could account for the effect of potential confounders. Where unbanded and fertile component Ca and P concentration and content were normally distributed, a mixed-effects multilevel regression model was used, with the predictor variables fertile status, egg mass, pond of origin, date of laying, and clutch. Size-matched pair was a second-level (grouping) variable.

5.4.5.4 Association between foetal and fertile egg component Ca and P content

To assess the association between foetal Ca (or P) content and that of the fertile egg's shell, shell membrane and yolk, a simple multi-variable regression model was used, with foetal Ca or P content as the outcome variable, and shell Ca content, shell membrane Ca (or P) content, yolk Ca (or P) content, foetal membrane Ca (or P) content, and total egg mass as the predictor variables.

5.5 Results

5.5.1 Assessment of accuracy of measurement

Since fertile egg samples were analysed together in a batch with unbanded egg samples, the same data were used for method and instrument calibration. Statistics describing accuracy of measurement (limit of detection, limit of quantification and standard reference material values) can be found in 4.5.1.

Duplicate shell and shell membrane samples showed the lowest variability in Ca concentration. Foetal fluids and membranes showed highly variable Ca and P concentration among duplicate samples (Table 5.1).

Table 5.1.
Summary of average of intra-assay coefficients of variation, expressed as percentages, of six duplicate measurements of Ca and P concentration in each fertile egg component

	Ca	P
Shell	0.24	—[a]
Shell membrane	1.57	4.43
Yolk	4.74	3.38
Foetus	3.38	2.45
Foetal fluids and membranes	18.81	15.79

[a] P was below detection limit in all shell samples

5.5.2 Masses of the components of size-matched unbanded and fertile eggs

Of the 30 size-matched unbanded and fertile egg pairs, mean within-pair difference in egg mass was 1.10 g (SD 1.26 g, min 0.001 g, max 4.98 g).

Mean masses of size-matched unbanded and fertile egg components are shown in Table 5.2.

Although the mean total mass of unbanded and fertile eggs was very similar, the mean wet mass of shell and the yolk of fertile eggs was noticeably lower than that of unbanded eggs.

Table 5.2.
Wet masses (g) of components of 30 pairs of Nile crocodile eggs, with each pair consisting of a fertile egg and an unbanded egg matched according to whole egg mass

	Unbanded		Fertile	
	Mean	SD	Mean	SD
Shell	13.44[a]	1.80	11.07	1.09
Shell membrane	1.98[b]	0.25	3.39	0.36
Yolk	46.73	9.47	17.62	4.75
Albumen	40.37	7.71	—	—
Foetus[c]	—	—	50.39	7.72
Foetal membrane and fluid	—	—	21.53	4.17
Whole egg	105.94	12.76	105.67	12.50

[a, b] Wet mass of unbanded shell and shell membranes were estimated (refer to Section 5.4.4)

[c] Mass of foetus after removing intra-abdominal yolk

5.5.3 Comparison of Ca or P concentration and content between size-matched pairs

Statistics describing unbanded and fertile Ca and P component concentrations are shown in Table 5.3, and those describing unbanded and fertile component Ca and P content are shown in Table 5.4.

As was seen for unbanded eggs, shell Ca concentration was highly consistent among observations. Shell membrane Ca concentration was normally distributed among fertile egg samples, in contrast to the highly positively skewed data obtained for unbanded egg samples. Yolk from unbanded eggs contained a higher mean concentration of Ca than yolk from fertile eggs. Foetal tissue contained a substantial concentration of Ca and P, with comparatively little variability between samples. The mixture of foetal membranes, foetal fluid and residual albumen contained a fair concentration of Ca and P that varied considerably compared to the foetal tissue.

Table 5.3.
Concentrations of Ca and P (in g/kg) in the components of unbanded and fertile Nile crocodile eggs

	Unbanded						Fertile					
	Mean	SD	Median	p25[a]	p75[b]	n	Mean	SD	Median	p25	p75	n
Ca												
Shell	392.62	4.23				3[c]	390.01	2.88				30
Shell membrane			17.33	9.99	34.47	29[d]	12.47	2.39				30
Yolk	7.72	0.33				30	6.02	1.92				30
Albumen			1.61	1.31	1.76	30						
Foetus							26.00	1.81				30
Foetal membrane and fluid							6.33	3.85				30
P												
Shell membrane			0.18	0.14	0.21	29[d]			0.09	0.08	0.10	30
Yolk	13.45	0.39				30	10.39	1.24				30
Albumen			0.66	0.53	0.76	30						
Foetus							17.72	0.97				30
Foetal membrane and fluid							4.52	1.52				30

[a] 25th percentile

[b] 75th percentile

[c] Only three unbanded shell samples were measured: refer to Section 5.4.4 above

[d] One unbanded shell membrane sample was excluded due to suspected contamination by shell

It is evident from Table 5.4 that most Ca in fertile eggs is contained in the shell and foetus, with a substantially smaller quantity in the yolk and shell membrane.

Table 5.4.
Mean Ca and P content (in grams) in size-matched pairs of unbanded and fertile eggs

	Unbanded					Fertile					
	Mean	SD	Median	p25[a]	p75[b]	Mean	SD	Median	p25	p75	n
Ca											
Shell[c]	4.38	0.60				4.16	0.43				30
Shell membrane[d]			0.03	0.01	0.05	0.02	0.004				29
Yolk[e]	0.23	0.04				0.05	0.02				16
Albumen[e]	0.03	0.009									16
Foetus						0.60	0.07				30
Foetal membrane and fluid								0.003	0.0026	0.005	30
Total egg	4.59	0.65				4.82	0.40				16
P											
Shell membrane			0.0003	0.0002	0.0003			0.0001	0.0001	0.0002	29
Yolk	0.40	0.07				0.08	0.02				16
Albumen	0.01	0.004									16
Foetus						0.41	0.05				30
Foetal membrane and fluid								0.003	0.002	0.004	30
Total egg	0.41	0.07				0.50	0.05				16

[a, b] 25th and 75th percentile

[c] Estimated shell Ca content based on mean shell concentration of 391.21 g/kg

[d] One unbanded shell membrane sample was excluded due to suspected contamination by shell

[e] Total dry yolk and albumen mass data is missing for 14 of 30 samples.

5.5.3.1 Total egg Ca content

Numerically, the average content of Ca in fertile eggs is 0.23 g higher than that in unbanded eggs, whereas the average content of P in fertile eggs is 0.09 g higher than that in unbanded eggs (Table 5.4).

However, if the effect of potential confounding factors were taken into account in a multilevel mixed-effects regression model, fertility status had no significant effect on Ca content ($P = 0.50$). The only factor affecting total egg Ca content was wet egg mass: for each increase by one gram in wet egg mass, total egg Ca content increased by 0.03 g (SE = 0.008, 95% CI = 0.012–0.042, $z = 3.56$, $P < 0.001$). The Wald's chi-square value for this model was 55.48 ($P < 0.001$, df = 8).

Fertility status was the only factor that had an effect on total egg P content. Fertile eggs contained on average 0.08 g more P than unbanded eggs (SE = 0.036, 95% CI = 0.015–0.15, $z = 2.37$, $P < 0.05$). The model's Wald's chi-square value was 69.87 ($P < 0.001$, df = 8).

5.5.3.2 Shell

Shell Ca concentration was measured for only three unbanded eggs of 30 pairs, so comparison between unbanded and fertile shell concentration cannot be made here. A mean Ca concentration was used to determine an estimated unbanded shell Ca content (refer to 5.4.4).

Without considering the effect of covariates, the measured mean Ca content of 30 fertile eggshells was 0.22 g (SD 0.55 g) less than the mean estimated Ca content of 30 eggshells from size-matched unbanded eggs. However, in the multilevel mixed effects regression model, egg mass was the only significant predictor of shell Ca content. For each one gram increase in egg mass, shell Ca content increased by 0.035 g (SE = 0.005, 95% CI = 0.024–0.045, $z = 6.47$, $P < 0.001$). A coefficient of −0.31 for fertility status suggested that, controlling for covariates, shells of fertile eggs are expected to have 0.31 g less Ca than those of unbanded eggs. However,

the effect of fertility status did not reach significance (P = 0.075). In line with this finding, Figure 5.1 shows that the Ca content of shells from fertile eggs were mostly, but not always, lower than that of the shells of unbanded eggs of similar mass. The Wald's chi-squared value of the model was 81.51, with P < 0.001, and df = 8. It can therefore be concluded that egg mass remains the principle determinant of shell Ca content, even among a mixture of unbanded and fertile eggs.

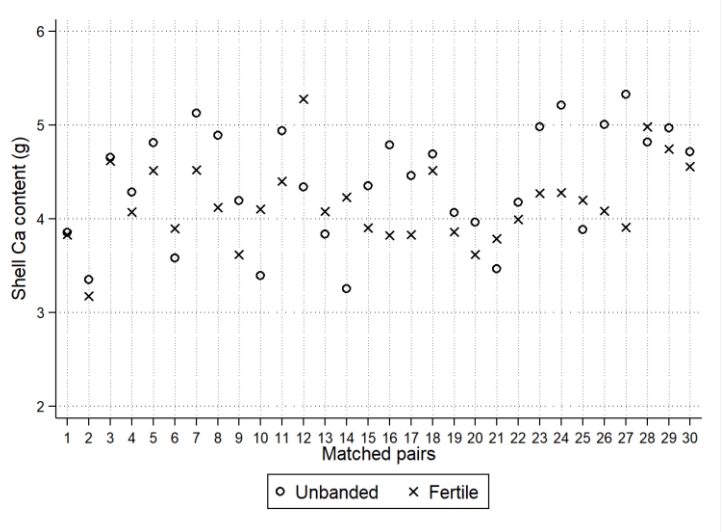


Figure 5.1.
Difference in measured shell Ca content of fertile eggs and estimated shell Ca content of size-matched unbanded eggs

5.5.3.3 Shell membrane

Shell membrane of unbanded eggs had relatively low Ca and P concentrations, and a relatively large variation in these values. The wet mass of the shell membrane of unbanded eggs was estimated from measurement of fertile eggs. Due to these potential methodological issues, the researchers are hesitant to speculate on differences in Ca and P content between unbanded and fertile shell membranes.

5.5.3.4 Yolk

The distribution of concentrations of yolk Ca and P in specimens of fertile eggs was noticeably wider than from those of corresponding size-matched unbanded eggs (Figure 5.2).

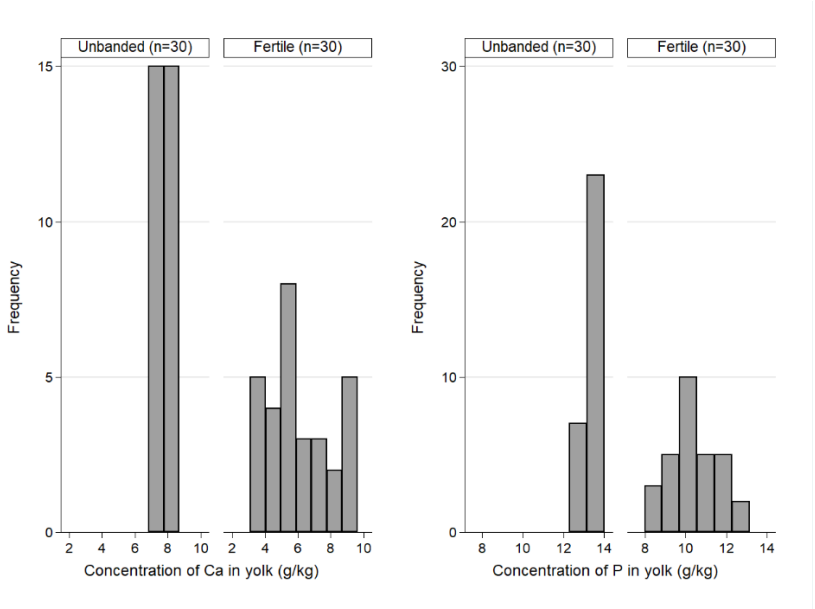


Figure 5.2.
Frequency distribution of yolk Ca and P concentration in unbanded and size-matched fertile eggs

In the mixed-effects regression model, it was found that the Ca concentration in the yolk of fertile eggs was on average 1.68 g/kg lower than that of unbanded eggs of similar size (SE = 0.64, 95% CI = −2.93 to −0.42, z = −2.62, P < 0.05). The model's Wald's chi-squared value was 35.41, with P < 0.001. As an exercise, if the effect of covariates other than fertility status (egg mass, pond, clutch, date of laying), which were either non-significant or had very small coefficients, was disregarded and the mean value of fertile yolk Ca concentration (6.02 g/kg) was simply subtracted from the mean value of unbanded yolk Ca concentration (7.72 g/kg), a very similar figure of 1.70 g/kg was obtained.

Using a mixed-effect regression model and after correcting for the possible effects of wet egg mass, pond of origin, clutch of origin and date of laying, the P concentration in the yolk of fertile eggs was on average 3.56 g/kg lower than that of unbanded eggs of similar size (SE = 0.41, 95% CI = −4.36 to −2.76, z = −8.69, P < 0.001). The model's Wald's chi-squared value was 252.80, with P < 0.001 and df = 8. If the effect of covariates was disregarded and the mean value of fertile yolk P concentration (10.39 g/kg) was subtracted from the mean value of unbanded yolk P concentration (13.45 g/kg), a similar figure of 3.06 g/kg was obtained.

After correcting for egg mass, yolk Ca concentration, pond of origin, date of laying and clutch of origin, the Ca content of yolks of 16 fertile eggs was on average 0.17 g lower than that of yolks of 16 unbanded eggs of similar size (SE = 0.01, 95% CI = −0.20 to −0.14, z = −11.50, P < 0.001) (The model's Wald's chi-squared value was 1587.34, with P < 0.001 and df = 9). As for yolk Ca concentration, if the effect of covariates was disregarded, and the mean value of fertile yolk Ca content (0.05 g) was subtracted from the mean value of unbanded yolk Ca content (0.23 g), a figure of 0.18 g was obtained, a value very similar to that found using the mixed-effect regression model. This difference can be seen in Figure 5.3.

Similarly, for yolk P content, fertile eggs had on average 0.26 g less P than unbanded eggs (SE = 0.03, 95% CI = −0.33 to −0.2, z = −8.27, P < 0.001) (The model's Wald's chi-squared

value = 1401.45, with P < 0.001, df = 9). If the mean value of fertile yolk P content was simply subtracted from the mean value of unbanded yolk P content, a figure of 0.32 g was obtained (Figure 5.3).

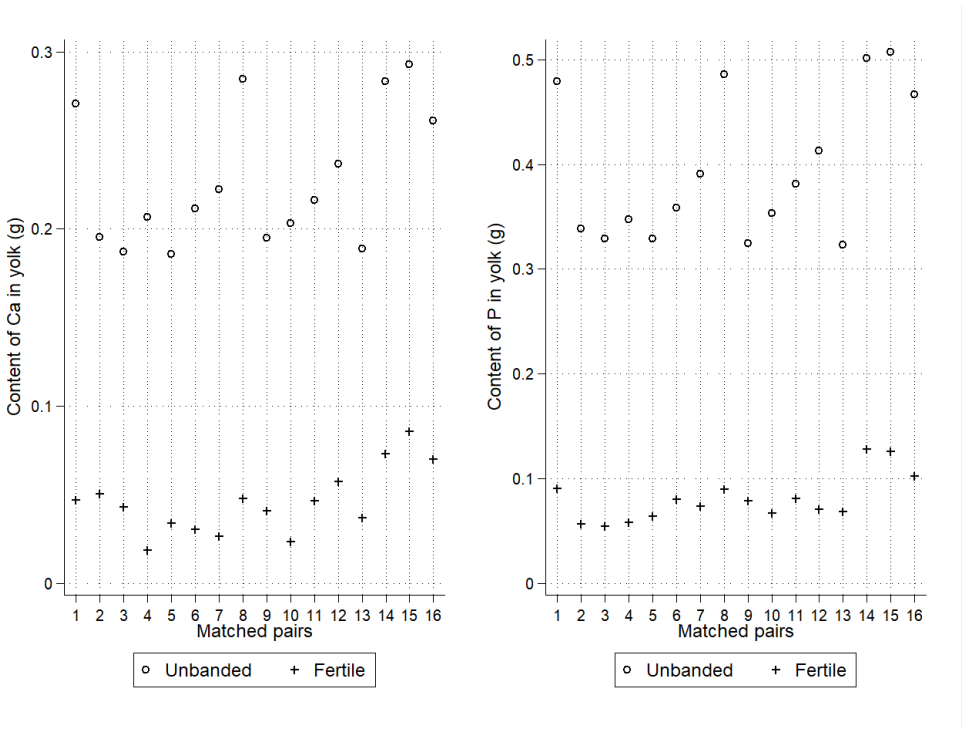


Figure 5.3.
Comparison of Ca and P content between size-matched pairs of fertile and unbanded egg yolks

5.5.4 Association between foetal Ca and P content and that of other fertile egg components

Using a multivariable regression model it was shown that the only predictor of foetal Ca content, was yolk Ca content. After removing non-significant interaction terms, for each gram that foetal calcium content increased, the yolk calcium content was expected to increase by a mean of 1.80 g (SE = 0.69, 95% CI = 0.39–3.22, t = 2.61, F(1, 28 df) = 6.83, r^2 = 0.20, P < 0.05).

After removing non-significant interaction terms, it was found that for each gram that foetal P content increased, the P content of a mixture of foetal membrane, foetal fluid and residual albumen decreased by a mean of 6.91 g (SE = 3.13, 95% CI = −13.32 to −0.50, t = −2.21, F(1, 28 df) = 4.87, r^2 = 0.15, P < 0.05).

5.6 Discussion

5.6.1 Within-sample variation

As seen in Table 5.1, the largely inorganic, homogeneous shell samples generated the most repeatable within-sample measurements, a situation similar to that seen for unbanded eggs (refer to 4.5.1). By contrast, the freeze- and oven-dried mixture of foetal membranes and foetal fluid was heterogeneous, and repeated analysis yielded highly variable results. It consisted of dried, leathery foetal membrane together with the powdery remnant of dried foetal fluids and residual albumen. Collecting a representative sample of this mixture was difficult. If future analysis of foetal membranes or fluids is desired, a possible solution to this issue would be to first collect the foetal fluid and residual albumen, then rinse the foetal membranes with deionised water prior to drying and analysis.

As discussed previously (refer to Section 2.5 of this thesis), it was far easier to separate the shell and shell membranes of fertile eggs than those of unbanded eggs, likely due to weakening of the attachment between the organic shell membrane and mammillary layers of the shell during incubation (Ferguson, 1982). This was reflected in the mean within-sample variability for shell membrane Ca, which was much higher for unbanded eggs (Table 4.1) than in fertile eggs (Table 5.1). Shell membranes of fertile eggs, which peeled away easily from their shells, were probably less susceptible to contamination by shell Ca than those of unbanded eggs.

Interestingly, repeated analyses of specimens taken from a particular fertile egg yolk varied more than those taken from a particular unbanded egg yolk. For duplicate unbanded egg

samples, the CV of yolk Ca concentration was 0.54% and for yolk P concentration it was 0.52% (Table 4.1), while for six duplicate sample pairs of fertile egg yolk the CV for Ca concentration was 4.74% and for P it was 3.38%. This could suggest that there is an uneven utilisation of yolk Ca and P from the yolk by the foetus. For yolk Ca, a possible alternative explanation is that the portion of yolk immediately beneath the yolk sac membrane has a higher concentration due to active local deposition of shell-derived Ca, as discussed by Packard and Packard (1989).

The developing foetus and recently-hatched neonate benefit from a store of energy-rich yolk phosphoglycerides, such as phosphatidylcholine and phosphatidylethanolamine (Noble *et al.*, 1991). Rather than having P uniformly bound up in such phosphoglycerides and distributed evenly through the yolk, data from the current study showed substantial variability in P concentration in paired specimens derived from the same fertile yolks, which suggested an uneven P distribution. Redistribution of yolk lipid may occur between yolk content and yolk sac membrane during incubation (Noble *et al.*, 1990, Noble and Moore, 1967), which may explain variability in P content from different sampling sites on the same yolk. A tentative alternative explanation is that there is simply some stratification of these compounds in the yolk based on their density, which is reflected in varying figures for P concentration.

5.6.2 Comparison of unbanded and fertile eggs

In selecting a size-matched unbanded egg to pair with each fertile egg, only the relative masses of the two eggs, and not clutch (female) of origin, breeding pond, or time within the laying season were considered, however these potential sources of variability were accounted for in the statistical models. In support of such an approach, it has been shown in previous work that unbanded yolk Ca and P content is principally determined by egg mass (Figure 4.1), and that larger eggs have a tendency to contain larger yolks in a linear fashion (refer to 3.5.5.2).

An alternative approach could have been to remove an egg from a clutch immediately after laying, arrest its development by chilling, and use it for direct within-clutch comparison with a fertile clutch mate near the point of hatching. If costs and commercial farming considerations do not constrain the purchase of a larger number of freshly laid fertile eggs, this approach may be easier to implement prospectively, since it has already been shown that eggs are more similar in mass within clutches than between clutches (Table 3.5). This would obviate the need to weigh many randomly selected unbanded eggs to find an optimally size-matched pair. Such an approach would also simplify statistical analysis by removing possible confounding factors. In previous reptilian egg research (Packard *et al.*, 1984, Packard and Packard, 1989), this method has been applied. Such a method also lends itself most easily to the study of eggs within wild nests.

5.6.2.1 Total egg Ca and P

Descriptive statistics indicated that the mean total Ca content of fertile eggs was numerically approximately 5% higher than that of size-matched unbanded eggs, and that of total fertile egg P approximately 20% higher than size-matched unbanded egg P (Table 5.4). However, when evaluated using a multilevel mixed-effects model, fertility status had no significant effect on Ca content: the only factor affecting total Ca content among a mixed sample of unbanded and fertile size-matched eggs was wet egg mass.

Fertility status did appear to affect egg P content: fertile eggs contained on average a fraction of a gram more P than unbanded eggs. This cannot be easily explained, but given that the difference was very small (less than 0.1 g) and the number of samples analysed was also small (16 matched pairs), caution should be exercised when attaching significance to this finding. Tissues containing P in the fertile egg may have been more amenable to digestion and analysis which reflected as higher P concentration and hence content. Another potential explanation is that this very difference in P content is an important determinant of the fertility status of the

egg. Such differences in P content could reflect variations in the type of lipoprotein molecules between unbanded and fertile eggs. However, if differences in lipoprotein composition were present which would affect embryonic development, one might expect such effects to manifest further along in development, resulting in death during incubation. The effect of lipoproteins on embryonic development in crocodilians requires further research, but a helpful overview of the topic in reptiles is provided by Thompson *et al.* (2004).

Simply by comparing content of Ca and P in unbanded and fertile components, it is evident that the principle source of Ca for the developing foetus is the shell and yolk, and for P, the yolk.

5.6.2.2 Shell Ca

In the present study, only three size-matched unbanded eggs were used to compare shell Ca concentration with that of fertile eggs, making the drawing of meaningful conclusions difficult. However, when the mean shell Ca concentration of 15 unbanded eggs used in the previous study (Table 4.2) is compared with that of the fertile eggs in the present study, there is very little apparent difference (unbanded eggs: mean 391.87 g/kg, SD 3.90, n = 15; fertile eggs: mean 390.01 g/kg, SD 2.88, n = 30). A likely explanation for this is that shell Ca is simply removed by the chorioallantois via enzymatic digestion of the innermost shell layers (Tuan *et al.*, 1986), without changing the remaining shell.

When comparing measured shell Ca content of fertile eggs with the estimated shell Ca content of unbanded eggs in a mixed-effects regression model, fertility status had no apparent effect, and the only predictor of total shell Ca was egg mass. However, on evaluation of Figure 5.1, it is evident that for 22 of 30 size-matched pairs, the shell of the unbanded egg contained a greater amount of Ca than the shell of the fertile egg. While all pairs of eggs consisted of members that were within 5 g of each other, it is possible that those pairs with a greater mass differential exerted a disproportionate effect on mean shell Ca content.

5.6.2.3 Shell membrane Ca and P

As discussed, due to methodological issues, unbanded egg shell membrane was likely an unrepresentative specimen, in contrast to that of fertile eggs, which was probably more representative. The shell membrane of fertile eggs contained a very low concentration of P, but a Ca concentration that was approximately double that of the yolk (Table 5.3), however total content of both elements was very low (Table 5.4). Since Ca must necessarily be moved from the shell across the shell membrane to the chorioallantois during the process of foetal development (Ferguson, 1985), it is possible that any Ca found in the shell membrane represents Ca "in transit" from shell to chorioallantois.

Since it was shown that the shell membrane contains negligible quantities of Ca, a possible improvement on the method used, would be to measure the combined shell and shell membrane Ca instead of trying to separate these two layers.

5.6.2.4 Yolk Ca and P

Figure 5.2 shows a wider distribution of Ca and P concentration among fertile egg yolks compared to unbanded egg yolks. For fertile yolk Ca, the spread in concentration is quite wide, with approximately as many fertile egg yolks displaying a Ca concentration of 3–4 g/kg as those displaying a concentration of 9–10 g/kg. A possible explanation for this is that uptake of Ca by the foetus from the yolk occurs in waves, which in turn are followed by waves of uptake of Ca from the shell by the CAM, stored temporarily in the yolk. If this is the case, then the histogram for yolk Ca concentration shown in Figure 5.2 is a snapshot of a dynamic process. Alternatively, the present findings could be mirroring those of a study by Packard and Packard (1989), which showed a sudden sharp increase in mean adjusted yolk Ca content in the last seven days of incubation in *A. mississippiensis* eggs. The temporal change in component Ca and P concentration and content of fertile eggs was not evaluated in the present study, so a direct comparison with the study by Packard and Packard (1989) is not possible. However,

among the samples used in the current study there was some variability in the time between projected hatching and the date of sample collection, so the spread in Ca concentration may be reflective of this. In the regression analyses, length of incubation was not identified as a statistically significant covariate, however the effect may have been small enough to escape notice, given the limited sample size.

The graphic of yolk P concentration seen in Figure 5.2 cannot be explained by 'uptake in waves' theory, since there is not a ready source of P outside of the yolk available to supplement yolk reserves of this element as they are incorporated into foetal tissue. Another possible explanation for the shape of the graphs for fertile egg Ca and P, is that there is a continuous exchange of Ca and P between yolk and foetus against a background of net transfer from the former to the latter. Packard and Clark (1996) noted that the flux of Ca across the yolk sac epithelium of avian embryos was bidirectional, but could not explain its mechanism of action.

Regardless of possible temporal changes in Ca and P concentration in fertile yolks, Figure 5.3 shows that there is clearly far less total Ca and P in fertile yolks than in size-matched unbanded yolks, indicating that the yolk is an important source of these elements for the developing foetus.

The Ca concentration in the yolk of fertile eggs was about the same or higher than that in the yolk of size-matched unbanded eggs, whereas P concentration in the yolk of fertile eggs was lower than in the yolk of size-matched unbanded eggs. It is probable that fertile yolk Ca content declines due to a reduction in yolk volume through incubation, while fertile yolk P content declines due to a reduction in both yolk volume and P concentration through incubation.

Packard and Packard (1989) found that the residual yolk of *A. mississippiensis* at hatching contained Ca and P in the ratio 2:1 — similar to that found in the skeleton. Data from the present study did not reflect such a ratio. However yolks were not sampled at precisely the

same interval prior to hatching, so it is possible that Ca uptake from the shell may have been in progress, and would have ultimately led to such a residual yolk Ca:P ratio by the time of hatching.

5.6.2.5 Ca and P in foetal membrane, foetal fluids and remnant albumen

As discussed, sample processing methodology for this component requires some alteration before reliable conclusions can be drawn. However, median figures indicate that net contribution of this mixture of components to overall Ca and P content is very small.

5.6.2.6 The contribution of albumen Ca and P to the foetus

Compared to yolk (for Ca and P) and shell (for Ca), the concentration and total amount of Ca and P in the unbanded egg albumen was very low, suggesting that this component contributed little to foetal Ca and P.

5.6.3 Conclusions

The present research describes Ca and P concentration and content in the components of the fertile Nile crocodile egg, which will allow for comparison with other crocodilian and reptilian species. Most Ca for foetal development is derived from the shell and yolk, and almost all P is derived from the yolk. The present study's findings support those of Packard and Packard (1989) in *A. mississippiensis*.

In future studies, shell and yolk are the samples that should be collected and analysed to assess the effect of management interventions, dietary alterations or fat-soluble toxic substances on crocodile egg Ca and P metabolism.

5.7 Acknowledgements

The researchers would like to thank Mr Stefan van As and Ms. Karon Griffiths for provision of sample material and assistance during data collection, as well as Mr Kgomotso Sekwele for assistance in egg processing.

5.8 Sources of funding

Funding for this research was provided by University of Pretoria in the form of a bursary to the first author, and by the South African National Research Foundation (NRF) in the form of an incentive fund for rated researchers to the last author (grant number 85836).

5.9 Declaration of conflicts of interest

None

Chapter 6. Summarising discussion

The study of crocodilian breeding has followed a trend that has mirrored the changing economic importance of these species over the past fifty years. Early research was performed by naturalists interested in understanding how crocodilian species interacted with their wild environment.

Recently, as a market for high quality crocodile leather has become established, more intensive research into crocodilian reproductive physiology has sought to address production- and husbandry-related issues confronting intensive commercial crocodile farmers.

Assessing the efficacy of interventions such as changes in nutrition, selection of breeding females or alteration of incubation regimens on egg composition or hatchling health, requires meticulous planning and execution of research trials. An essential component of any research study is data collection, which requires time and often substantial amounts of money. Where crocodile eggs or neonates are concerned, the seasonal nature of crocodile breeding means that samples are available once a year, so a missed opportunity often requires postponement of sample collection to the following year, with all the associated disadvantages that this imposes. For this reason, embarking on sample collection with a clear vision of the type and number of specimens required as well as potential pitfalls in sample collection, confers a substantial advantage to the researcher.

To briefly summarise the preceding chapters of this thesis, in Chapter 3 the variation in masses of unbanded and fertile *C. niloticus* eggs and their components was described, together with the percentage contribution of each component to total egg mass. Egg mass was related to estimated egg volume, the effect of potential covariates on the mass of the egg and its various components was described, and the grouping effect of clutch on the masses of unbanded and fertile egg components was assessed. In Chapters 4 and 5 the Ca and P concentration and

content of each component of unbanded and fertile eggs was measured and the grouping effect of clutch on these variables in unbanded eggs was assessed. The effect of potential confounding factors that may have prevailed on this particular farm on Ca and P concentration and content in unbanded and fertile eggs were evaluated, and the Ca and P concentration and content of size-matched unbanded and fertile eggs were compared.

Knowledge gained from this research should have application in further research into captive hatchling production as well as into conservation and assessment of the reproductive health of wild populations. As one of the first in-depth studies of Ca and P in Nile crocodile eggs, the present research was necessarily descriptive. It allowed the development of skills transferrable to the study of other inorganic as well as organic egg constituents, and provided a stimulus for the devising of new research questions.

All egg samples used in the present research were derived from the same farm, a fact which conferred both advantages and disadvantages to the data and its interpretation. While it cannot be claimed (or even implied) that data from these studies are representative of all Nile crocodiles everywhere, the fact that samples were handled in a similar manner, came from females kept under similar conditions and fed a similar diet should allow for the drawing of conclusions about natural variations within a population.

Through trial and error, lessons were learned about how to (and how not to) dissect crocodile eggs. Eggs gradually lose mass through transpiration during storage. This can be managed by processing the eggs soon after collection, keeping them as cool as possible by refrigeration, and by storing them separately in small sealed plastic bags to reduce water loss.

The albumen clarity score (Section 2.2) is an important consideration when selecting samples. In the studies described in this thesis, it was found that most unbanded egg samples were unsuitable due to putrefaction or coagulation of yolk and albumen. The use of the clarity score

allowed these eggs to be identified immediately and discarded. Unbanded eggs were removed at the end of the incubation period once all fertile eggs had hatched. Had they been removed immediately or within a few days of laying, eggs would have been fresher at opening, and a greater proportion may have had an albumen clarity score of one.

The antibacterial effects of albumen proteins in birds' eggs have been discussed by Burley *et al.* (1987), who found no such proteins in albumen of *Crocodylus porosus*. Instead, the researchers suggested that the thick crocodilian shell membrane forms the main barrier against microbial invasion. The immune function of the shell membrane, particularly during development of the chorioallantois in the fertile egg, represents an interesting avenue of further research.

Separation of shell and shell membrane in unbanded eggs was difficult, reflecting the findings of Kern and Ferguson (1997). The process of separation in unbanded eggs so severely compromised the quality of the resulting shell membrane samples, that reliable conclusions could not be drawn on their Ca and P content. In future research, such mechanical separation should be avoided. If chemical analysis must be performed on unbanded eggshells or membranes, they must either be analysed together, or the shell must be removed from the shell membrane by dissolution in a weak acid. The same difficulty does not exist when separating the shells of fertile shells and shell membranes.

As discussed in Chapter 1, maternally derived factors transmitted to the foetus through the egg may affect the pre- and post-hatching health of the embryo, foetus or hatchling. The way the egg is handled during the incubation period may also influence hatchling health. Moreover, genetic traits of male origin transmitted to the foetus could conceivably affect foetal size, growth rate or health. Where multiple sires are available, the possibility of superfecundation (polyandry) introduces yet another confounding variable that must be accounted for in study designs.

Webb *et al.* (1983b) found that *Crocodylus johnstoni* clutches laid at the beginning of the laying season tended to contain greater numbers of larger eggs. In the current study no such association was found in captive Nile crocodiles. However, Webb *et al.* (1983b) evaluated a wild population, while the present research was confined to captive-bred animals on a single farm. The environmental pressures on a wild population are in all likelihood vastly different to the stresses of animals in captivity, so it may be unwise to categorically state that the time within the laying season has no effect on *C. niloticus* clutch or egg size.

Although each clutch was not traced to a particular female, a breeding pond containing females that were on average larger and more mature, was also the source of the largest eggs, a finding consistent with prior research (Thorbjarnarson and Hernández, 1993, Huchzermeyer, 2003, Deitz and Hines, 1980). An inverse relationship was found between egg mass and the number of eggs in a clutch (Section 3.5.5). This contrasted with the findings of Deitz and Hines (1980) for *Alligator mississippiensis* and Thorbjarnarson and Hernández (1993) for *C. intermedius*, who found positive relationships between clutch size and egg mass.

Nile crocodile eggs are not perfectly ellipsoid and may vary considerably in shape. This was discussed in detail by Nöthling *et al.* (2019a), who devised a mathematical method for determining the original egg volume from a photograph of an egg remnant found in the nest after hatching. In research described in Chapter 3 of this thesis, it was found that the volume of an egg, estimated as its ellipsoid volume, correlated closely with its mass. If mass and volume can be measured or accurately estimated, then density can be calculated. Variations in the density of eggs from different populations could imply variations in shell thickness or the ratio of lipid to water of the egg content, which may in turn have implications for the nutrition of the developing embryo.

Controlling for egg mass, there was a positive correlation between incubation time and the combined mass of the foetus and its intra-abdominal yolk, suggesting that foetal growth was

occurring even in late incubation. All foetuses examined during the last ten days of incubation had internalised their yolk, which suggested that they were probably in the late-incubation plateau phase of the sigmoid development curve described by Whitehead *et al.* (1990). Incubation period had no effect on the isolated intra-abdominal yolk mass. Since all eggs were incubated at the same temperature, this may have reflected either a natural variability in yolk mass among egg specimens, or a temporary suspension of yolk uptake by the foetus in late incubation. The apparent gain in mass of late-incubation foetuses observed may have been due to the uptake of water from residual albumen or foetal fluids. Given the relatively small sample size (n = 30), it may also have been a coincidental, as Nöthling *et al.* (2019b) found that for a particular egg volume, hatchling mass may vary.

The mass of the intra-abdominal yolk of the foetus was loosely correlated with the egg in which it was contained (Section 3.5.2). The grouping effect of clutch on foetal or hatchling mass was not investigated, however findings of the present research appear to support those of Brien *et al.* (2014), who found that clutch had a substantial grouping effect on *Crocodylus porosus* hatchling mass. In further support of these findings, Nöthling *et al.* (2019b) recently found that hatchling mass varied substantially for a given egg volume but tended to be fairly consistent within clutches.

Prior researchers have explored the relationships between foetal size, the quantity of intra-abdominal yolk at hatching, stresses during incubation and post-hatching growth rate in crocodilians, and have shown conflicting results (Piña *et al.*, 2007, Hutton, 1987, Allsteadt and Lang, 1995). Hutton (1987), in his evaluation of the effect of incubation temperature on sex determination in *C. niloticus*, did not specifically measure the mass of intra-abdominal yolk (he did not sacrifice any hatchlings) however he did speculate that smaller hatchlings could carry a greater quantity of abdominal yolk. The current research found that egg mass correlated strongly with hatchling mass (confirming the findings of Deeming and Ferguson (1989)), and

egg mass correlated loosely with intra-abdominal yolk mass, but contrary to Hutton's suggestion (Hutton, 1987), hatchling mass did not correlate with intra-abdominal yolk mass.

Clutch was shown to have a strong grouping effect on the mass of unbanded eggs, as well as that of their components (Section 3.5.4). Furthermore, unbanded egg yolk mass varied little within clutches relative to between clutches (Table 3.5 and Figure 3.6). Subsequent to the data collection and analysis presented in this thesis, Nöthling *et al.* (2019b) found that the productivity of an egg (defined as hatchling mass relative to egg volume) varied slightly more within clutches than between clutches (55% of variation occurred among eggs of the same clutch and 45% among eggs from different clutches).

If the findings of the present study are combined with those of Nöthling *et al.* (2019b), it appears likely that there should be a greater variability in egg productivity within a clutch, where the yolk mass varies less, than between clutches, where the yolk mass varies more. The influence of initial yolk mass on foetal mass (and hence egg productivity) requires further investigation. Finkler *et al.* (1998) found that aspiration of 20% of yolk mass from chicken eggs resulted in no significant difference in hatchling mass or the length of long bones, compared to those from sham-aspirated eggs. Whether the extremely fragile perivitelline membrane of the Nile crocodile egg lends itself to aspiration of its content as described by Finkler *et al.* (1998) remains to be seen. An alternative methodology to that of Finkler *et al.* (1998) to investigate the role of initial egg yolk mass on hatchling mass may involve the sacrificing of a certain number of eggs within a clutch immediately after laying and assessing the correlation between the mean within-clutch yolk mass and mean within-clutch hatchling mass. In such a study, foetal size and growth rate may be influenced by sire-derived genetic traits, so single-sire breeding or genetic testing would be needed to eliminate superfecundation as a source of variability.

The effect of a given yolk mass for a given egg size on post-hatching health and growth has also not been investigated. Brien *et al.* (2014) found for *C. porosus* that the failure-to-thrive syndrome appeared to be heavily influenced by clutch and could not be explained by clutch variables such as egg size. Assuming all clutches were managed in the same manner, this could suggest that unexplained maternal factors were at play. A possible approach to investigating the effect of such factors on clutch viability would be to determine whether specific females repeatedly lay clutches containing a higher-than-average proportion of weak, abnormal or poorly growing hatchlings. These females could then be culled and carefully examined. On farms with large communal ponds containing many breeding females, it is presently very difficult to identify specific females and match them to their clutches. Such investigation would be best carried out under conditions where females are kept in small groups.

The Ca and P content of each component of the unbanded egg were primarily affected by the mass of that component, except for albumen, which was affected principally by each element's concentration (Section 4.5.2). In fertile eggs within ten days of hatching, most Ca was contained in the shell and foetus, while most P was contained in the yolk and foetus (Section 5.5.3). A sample of fertile eggs had a wider spread of yolk Ca and P concentration than did a sample of size-matched unbanded eggs, whose yolk Ca and P concentrations tended to fall within a narrow range (Figure 5.2). As discussed in 5.6.2, this could reflect the dynamic process of transfer of Ca (and P) between yolk, foetus and, in the case of Ca, shell. As with egg mass and egg component mass, clutch also had a strong effect on yolk Ca and P concentration and the Ca and P content of all unbanded components, with the exception of albumen. This fact is important for the design of future studies. Where total sample numbers are constrained by factors such as time or cost, studies should be designed by selecting a low number of eggs from as large a number of clutches as possible. Each clutch is laid by only one genetically distinct female, who in turn is subject to an array of environmental, managerial, social and nutritional

stresses that may vary from year to year. It is therefore logical that clutch, rather than female, is the lowest order egg grouping variable.

Chapter 7. Concluding statements

The crocodile egg is a perfectly packaged, self-contained reproductive unit that, together with its clutchmates, is the representative of the parent animals' contribution to the following generation. It is also considerably easier to study than its famously intractable parents.

For this thesis, a general, yet detailed description of the relationship between the components of unbanded and fertile Nile crocodile eggs was made. Although prior work on this topic has been done, these studies lacked particular focus on the relationship between egg components.

Given that captive reptiles are susceptible to disorders of Ca and P metabolism, a topic poorly researched in the crocodilians, the present research into Ca and P in the Nile crocodile egg provides a potential reference for researchers and clinical veterinarians who may suspect a disorder of Ca or P metabolism.

Chapter 8. References

ACKERMAN, R. & LOTT, D. 2004. Thermal, hydric and respiratory climate of nests In: Deeming DC, editor. *Reptilian Incubation. Environment, Evolution and Behaviour*. Nottingham University Press. Nottingham, UK.

AHN, D., KIM, S. & SHU, H. 1997. Effect of egg size and strain and age of hens on the solids content of chicken eggs. *Poultry Science,* 76**,** 914-919.

AKINS, R. E. & TUAN, R. S. 1993a. Transepithelial calcium transport in the chick chorioallantoic membrane. I. Isolation and characterization of chorionic ectoderm cells. *Journal of Cell Science,* 105**,** 369-379.

AKINS, R. E. & TUAN, R. S. 1993b. Transepithelial calcium transport in the chick chorioallantoic membrane. II. Compartmentalization of calcium during uptake. *Journal of Cell Science,* 105**,** 381-388.

ALDERTON, D. & TANNER, B. 1991. *Crocodiles & Alligators of the World*, Facts on file.

ALLSTEADT, J. & LANG, J. W. 1995. Incubation temperature affects body size and energy reserves of hatchling American alligators (*Alligator mississippiensis*). *Physiological Zoology,* 68**,** 76-97.

AMAVET, P., ROSSO, E., MARKARIANI, R. & PIÑA, C. I. 2008. Microsatellite DNA markers applied to detection of multiple paternity in *Caiman latirostris* in Santa Fe, Argentina. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* 309**,** 637-642.

ANDREWS, R. 2004. Patterns of embryonic development. In Deeming DC, editor. *Reptilian Incubation: environment, evolution and behaviour***.** Nottingham University Press. Nottingham, UK.

ARUKWE, A., MYBURGH, J., LANGBERG, H. A., ADEOGUN, A. O., BRAA, I. G., MOEDER, M., SCHLENK, D., CRAGO, J. P., REGOLI, F. & BOTHA, C. 2016. Developmental alterations and endocrine-disruptive responses in farmed Nile crocodiles (*Crocodylus niloticus*) exposed to contaminants from the Crocodile River, South Africa. *Aquatic Toxicology,* 173**,** 83-93.

AULIE, A. & KANUI, T. I. 1995. Oxygen consumption of eggs and hatchlings of the Nile crocodile (*Crocodylus niloticus*). *Comparative Biochemistry and Physiology--Part A: Physiology,* 1**,** 99-102.

BEBOUT, D. E. & HEMPLEMAN, S. C. 1994. Calcium deficient diet, acetazolamide and gas exchange characteristics of avian eggshells. *Respiration Physiology,* 95**,** 11-20.

BIGALKE, R. 1931. 29. Note on the egg of the Nile Crocodile (*Crocodylus niloticus*). *Journal of Zoology,* 101**,** 557-559.

BLAKE, D. & LOVERIDGE, J. 1975. The role of commercial crocodile farming in crocodile conservation. *Biological Conservation,* 8**,** 261-272.

BOCK, J. L., WOODWARD, A., LINDA, S., PERCIVAL, H. & CARBONNEAU, D. 2004. Hatching success of American alligator eggs when subjected to simulated collection trauma. *Proceedings of the Annual Conference of the Southeast Association of Fish and Wildlife Agencies*. 323-335.

BOUWMAN, H. 2004. South Africa and the Stockholm Convention on persistent organic pollutants: science policy. *South African Journal of Science,* 100**,** 323-328.

BOUWMAN, H., SEREDA, B. & MEINHARDT, H. 2006. Simultaneous presence of DDT and pyrethroid residues in human breast milk from a malaria endemic area in South Africa. *Environmental Pollution,* 144**,** 902-917.

BRIEN, M. L., WEBB, G. J., MCGUINNESS, K. & CHRISTIAN, K. A. 2014. The relationship between early growth and survival of hatchling saltwater crocodiles (*Crocodylus porosus*) in captivity. *PloS One,* 9**,** e100276.
BROCHU, C. A. 2003. Phylogenetic approaches toward crocodylian history. *Annual Review of Earth and Planetary Sciences,* 31**,** 357-397.
BROWN, G., FORBES, P., MYBURGH, J. & NÖTHLING, J. 2019. Nile crocodile (Crocodylus niloticus): Egg mass relative to egg component mass in unbanded and fertile eggs. *Aquaculture,* 505**,** 423–430.
BULL, J. J. & VOGT, R. C. 1979. Temperature-dependent sex determination in turtles. *Science,* 206**,** 1186-1188.
BURLEY, R., BACK, J., WELLINGTON, J. & GRIGG, G. 1987. Proteins of the albumen and vitelline membrane of eggs of the estuarine crocodile, *Crocodylus porosus*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 88**,** 863-867.
BURLEY, R., BACK, J., WELLINGTON, J. & GRIGG, G. 1988. Proteins and lipoproteins in yolk from eggs of the estuarine crocodile (*Crocodylus porosus*); a comparison with egg yolk of the hen (*Gallus domesticus*). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 91**,** 39-44.
CALDWELL, J. 2017. World trade in crocodilian skins 2013-2015. *United Nations Environment Programme–World Conservation Monitoring Centre Report.*
CARAFOLI, E. 1987. Intracellular calcium homeostasis (abstract only). *Annual Review of Biochemistry,* 56**,** 395-433.
CARDEILHAC, P. T., WINTERNITZ, D. L., J.D., B., O.L., B. & WOLFF, W. F. 1999. The moving median method to estimate normal egg parameters for alligator populations in the southern Everglades, Southwest Louisiana, and Lake Griffin. *IAAAM Proceedings,* 30**,** 155–159.
CAREY, C., RAHN, H. & PARISI, P. 1980. Calories, water, lipid and yolk in avian eggs. *The Condor,* 82**,** 335-343.
CARRUTHERS, J. 2008. Wilding the farm or farming the wild? The evolution of scientific game ranching in South Africa from the 1960s to the present. *Transactions of the Royal Society of South Africa,* 63**,** 160-181.
CEDILLO-LEAL, C., SIMONCINI, M. S., LEIVA, P. M., LARRIERA, A., LANG, J. W. & PINA, C. I. 2017. Eggshell structure in *Caiman latirostris* eggs improves embryo survival during nest inundation. *Proceedings of the Royal Society B: Biological Sciences,* 284**,** 20162675.
CHABRECK, R. H. 1978. Collection of American alligator eggs for artificial incubation. *Wildlife Society Bulletin (1973-2006),* 6**,** 253-256.
CHEN, S., FRANCIS, B. M. & DZIUK, P. 1993. Effect of concentration of mixed-function oxidase on concentration of estrogen, rate of egg lay, eggshell thickness, and plasma calcium in laying hens. *Journal of Animal Science,* 71**,** 2700-2707.
CHRISTIANS, J. K. 2002. Avian egg size: variation within species and inflexibility within individuals. *Biological Reviews,* 77**,** 1-26.
COLEMAN, J. R. & TEREPKA, A. R. 1972. Fine structural changes associated with the onset of calcium, sodium and water transport by the chick chorioallantoic membrane. *The Journal of Membrane Biology,* 7**,** 111-127.
COTT, H. B. 1961. Scientific results of an inquiry into the ecology and economic status of the Nile crocodile (*Crocodilus niloticus*) in Uganda and Northern Rhodesia. *The Transactions of the Zoological Society of London,* 29**,** 211-356.
DA SILVEIRA, R., FARIAS, I. P., MAGNUSSON, W. E., MUNIZ, F. L., HRBEK, T. & CAMPOS, Z. 2011. Multiple paternity in the black caiman (*Melanosuchus niger*)

population in the Anavilhanas National Park, Brazilian Amazonia. *Amphibia-Reptilia,* 32**,** 428-434.
DACKE, C., ELSEY, R., TROSCLAIR, P., SUGIYAMA, T., NEVAREZ, J. & SCHWEITZER, M. 2015. Alligator osteoderms as a source of labile calcium for eggshell formation. *Journal of Zoology,* 297**,** 255-264.
DAVENPORT, J., GROVE, D., CANNON, J., ELLIS, T. & STABLES, R. 1990. Food capture, appetite, digestion rate and efficiency in hatchling and juvenile *Crocodylus porosus*. *Journal of Zoology,* 220**,** 569-592.
DAVIS, L. M., GLENN, T. C., ELSEY, R. M., DESSAUER, H. C. & SAWYER, R. H. 2001. Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*. *Molecular Ecology,* 10**,** 1011-1024.
DEEMING, D. & FERGUSON, M. 1989. Effects of incubation temperature on growth and development of embryos of *Alligator mississippiensis*. *Journal of Comparative Physiology B,* 159**,** 183-193.
DEEMING, D. & FERGUSON, M. 1990. Morphometric analysis of embryonic development in *Alligator mississippiensis, Crocodylus johnstoni* and *Crocodylus porosus*. *Journal of Zoology,* 221**,** 419-439.
DEEMING, D. C. 1991. Reasons for the dichotomy in egg turning in birds and reptiles. *Egg incubation: its effects on embryonic development in birds and reptiles***,** 307-323.
DEITZ, D. C. & HINES, T. C. 1980. Alligator nesting in north-central Florida. *Copeia***,** 249-258.
DOHOO, I., MARTIN, W. & STRYHN, H. 2009. Veterinary Epidemiologic Research, 2nd. *VER Inc, Charlottetown, Prince Edward Island*.
DZIALOWSKI, E. M. & SOTHERLAND, P. R. 2004. Maternal effects of egg size on emu *Dromaius novaehollandiae* egg composition and hatchling phenotype. *Journal of Experimental Biology,* 207**,** 597-606.
DZOMA, B., SEJOE, S. & SEGWAGWE, B. 2008. Commercial crocodile farming in Botswana. *Tropical Animal Health and Production,* 40**,** 377-381.
ELSEY, R. & WINK, C. 1986. The effects of estradiol on plasma calcium and femoral bone structure in alligators. *Comp Biochem Physiol A,* 84**,** 107-110.
FERGUSON, M. W. 1981. Extrinsic microbial degradation of the alligator eggshell. *Science,* 214**,** 1135-1137.
FERGUSON, M. W. 1982. The structure and composition of the eggshell and embryonic membranes of Alligator mississippiensis. *The Transactions of the Zoological Society of London,* 36**,** 99-152.
FERGUSON, M. W. 1985. Reproductive biology and embryology of the crocodilians. *Biology of the Reptilia,* 14**,** 329-491.
FERGUSSON, R. A. 2010. Nile crocodile *Crocodylus niloticus*. *Crocodiles. Status Survey and Conservation Action Plan, 3rd edn (eds Manolis SC, Stevenson C)***,** 84-89.
FINKLER, M., VAN ORMAN, J. & SOTHERLAND, P. 1998. Experimental manipulation of egg quality in chickens: influence of albumen and yolk on the size and body composition of near-term embryos in a precocial bird. *Journal of Comparative Physiology B,* 168**,** 17-24.
FORD, N. B. 1989. Relationships among body size, clutch size, and egg size in three species of oviparous snakes. *Herpetologica,* 45**,** 75-83.
GAINES, P. R. 2011. ICP Operations Guide. *A guide for using ICP-OES and ICP-MS: Inorganic Ventures***,** 4-8.
GARNETT, S. & MURRAY, R. 1986. Parameters affecting the growth of the estuarine crocodile, *Crocodylus porosus,* in Captivity. *Australian Journal of Zoology,* 34**,** 211-223.

GARRISON, J. C. & TEREPKA, A. R. 1972. Calcium-stimulated respiration and active calcium transport in the isolated chick chorioallantoic membrane. *The Journal of Membrane Biology,* 7**,** 128-145.
GÖTH, A. & BOOTH, D. T. 2005. Temperature-dependent sex ratio in a bird. *Biology Letters,* 1**,** 31-33.
GRAHAM, A. D. 1968. The Lake Rudolf Crocodile *(Crocodylus Niloticus* Laurenti*):* Population, Wildlife Services Limited.
GREER, A. E. 1975. Clutch size in crocodilians. *Journal of Herpetology***,** 319-322.
GRIGG, G. & KIRSHNER, D. 2015. *Biology and evolution of crocodylians,* CSIRO PUBLISHING.
GRIGG, G. C. 1987. Water relations of crocodilian eggs: management considerations. *In:* WEBB, G., MANOLIS, S. C. & WHITEHEAD, P. J. (eds.) *Wildlife Management. Crocodiles and Alligators.* Chipping Norton: Surrey Beatty & Sons.
GUILLETTE, L. J., WOODWARD, A. R., YOU-XIANG, Q., COX, M. C., MATTER, J. M. & GROSS, T. S. 1995. Formation and regression of the corpus luteum of the American alligator (*Alligator mississippiensis*). *Journal of Morphology,* 224**,** 97-110.
HICKEY, J. J. & ANDERSON, D. W. 1968. Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. *Science,* 162**,** 271-273.
HINCKE, M. T., NYS, Y., GAUTRON, J., MANN, K., RODRIGUEZ-NAVARRO, A. B. & MCKEE, M. D. 2012. The eggshell: structure, composition and mineralization. *Front Biosci,* 17**,** 80.
HOLM, L., BLOMQVIST, A., BRANDT, I., BRUNSTRÖM, B., RIDDERSTRÅLE, Y. & BERG, C. 2006. Embryonic exposure to *o, p′*-DDT causes eggshell thinning and altered shell gland carbonic anhydrase expression in the domestic hen. *Environmental Toxicology and Chemistry: An International Journal,* 25**,** 2787-2793.
HUCHZERMEYER, F. W. 2003. *Crocodiles: biology, husbandry and diseases*, CABI.
HUTTON, J. 1987. Incubation temperatures, sex ratios and sex determination in a population of Nile crocodiles (*Crocodylus niloticus*). *Journal of Zoology,* 211**,** 143-155.
ISBERG, S., COMBRINK, X., LIPPAI, C. & BALAGUERA-REINA, S.A. . 2019. *Crocodylus niloticus. The IUCN Red List of Threatened Species 2019* [Online]. Available: https://www.iucnredlist.org/species/45433088/3010181 [Accessed 20 January 2020].
ISBERG, S., SHILTON, C. & THOMSON, P. 2009. Improving Australia's crocodile industry productivity: understanding runtism and survival. Rural Industries Research and Development Corporation.
ISBERG, S., THOMSON, P., NICHOLAS, F., BARKER, S. & MORAN, C. 2005. Quantitative analysis of production traits in saltwater crocodiles (*Crocodylus porosus*): II. Age at slaughter. *Journal of Animal Breeding and Genetics,* 122**,** 370-377.
JENKINS, N. 1975. Chemical composition of the eggs of the crocodile (*Crocodylus novaeguineae*). *Comparative Biochemistry and Physiology Part A: Physiology,* 51**,** 891-895.
JENKINS, R. W., JELDEN, D., WEBB, G. J. & MANOLIS, S. C. 2004. Review of crocodile ranching programmes. *Conducted for CITES by the Crocodile Specialist Group of IUCN/SSC. IUCN SSC Crocodile Specialist Group, Sanderson, Australia*.
JOANEN, T. & MCNEASE, L. 1975. Notes on the reproductive biology and captive propagation of the American alligator. Proceedings of the Annual Conference of the Southeastern Associated Game and Fish Committee. 407-415.

JOANEN, T. & MCNEASE, L. 1977. Artificial incubation of alligator eggs and post hatching culture in controlled environmental chambers. Proceedings of the annual meeting-World Mariculture Society. Wiley Online Library, 483-490.
JOANEN, T. & MCNEASE, L. 1987. *Alligator farming research in Louisiana, USA*, Surrey Beatty & Sons.
JOANEN, T., MCNEASE, L. & FERGUSON, M. 1987. The effects of egg incubation temperature on post-hatching growth of American alligators. *Wildlife Management: Crocodiles and Alligators.* Chipping Norton, Australia: Surrey Beatty and Sons (Pty Ltd.). 533-537.
KERN, M. D. & FERGUSON, M. W. 1997. Gas permeability of American alligator eggs and its anatomical basis. *Physiological and Biochemical Zoology,* 70**,** 530-546.
KHOSA, P., IMBAYARWO-CHIKOSI, V. E. & HAMANDISHE, V. 2012. Comparative analysis of hatching rates and clutch sizes of Nile crocodile (*Crocodylus niloticus*) eggs collected on-and off-farm in Zimbabwe. *Tropical Animal Health and Production,* 44**,** 905-909.
KILLIP, S., MAHFOUD, Z. & PEARCE, K. 2004. What is an intracluster correlation coefficient? Crucial concepts for primary care researchers. *The Annals of Family Medicine,* 2**,** 204-208.
KOFRON, C. P. 1990. The reproductive cycle of the Nile crocodile (*Crocodylus niloticus*). *Journal of Zoology,* 221**,** 477-488.
LANCE, S., TUBERVILLE, T., DUECK, L., HOLZ-SCHIETINGER, C., TROSCLAIR III, P., ELSEY, R. & GLENN, T. 2009a. Multiyear multiple paternity and mate fidelity in the American alligator, *Alligator mississippiensis*. *Molecular Ecology,* 18**,** 4508-4520.
LANCE, V., JOANEN, T. & MCNEASE, L. 1983. Selenium, vitamin E, and trace elements in the plasma of wild and farm-reared alligators during the reproductive cycle. *Canadian Journal of Zoology,* 61**,** 1744-1751.
LANCE, V. A. 1989. Reproductive cycle of the American alligator. *American Zoologist,* 29**,** 999-1018.
LANCE, V. A., ROSTAL, D. C., ELSEY, R. M. & TROSCLAIR III, P. L. 2009b. Ultrasonography of reproductive structures and hormonal correlates of follicular development in female American alligators, *Alligator mississippiensis*, in southwest Louisiana. *General and Comparative Endocrinology,* 162**,** 251-256.
LANE, T., BOYCE, W., REINHARD, M., BUERGELT, D., LARSEN, R., POULOS, P., KING, M. & CARDEILHAC, P. 1984. Disease Problems in Farm-Raised Hatchling Alligators on Florida Alligator Farms IAAAM 1984.
LANG, J. W. & ANDREWS, H. V. 1994. Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology,* 270**,** 28-44.
LAWRENCE, E. 2008. *Henderson's dictionary of biology,* Pearson education.
LEIVA, P., LABAQUE, M., FERNANDEZ, M., PIÑA, C. & SIMONCINI, M. 2018. Physical and chemical characteristics of fertile and infertile eggs of wild *Caiman latirostris*. *Aquaculture,* 497**,** 287-291.
LEWIS, J. L., FITZSIMMONS, N. N., JAMERLAN, M. L., BUCHAN, J. C. & GRIGG, G. C. 2013. Mating systems and multiple paternity in the estuarine crocodile (*Crocodylus porosus*). *Journal of Herpetology,* 47**,** 24-33.
LUNDHOLM, C. 1990. The eggshell thinning action of acetazolamide; relation to the binding of Ca2+ and carbonic anhydrase activity of the shell gland homogenate. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 95**,** 85-89.
LUNDHOLM, C. 1993. Inhibition of prostaglandin synthesis in eggshell gland mucosa as a mechanism for *p, p'*-DDE-induced eggshell thinning in birds—a comparison of ducks

and domestic fowls. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 106**,** 389-394.
LUNDHOLM, C. 1997. DDE-induced eggshell thinning in birds: effects of *p, p'*-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 118**,** 113-128.
LUTZ, P., BENTLEY, T., HARRISON, K. & MARSZALEK, D. 1980. Oxygen and water vapour conductance in the shell and shell membrane of the American crocodile egg. *Comparative Biochemistry and Physiology Part A: Physiology,* 66**,** 335-338.
MANOLIS, S., WEBB, G. & DEMPSEY, K. 1987. Crocodile egg chemistry. *In:* WEBB, G., MANOLIS, S. & WHITEHEAD, P. (eds.) *Wildlife Management: Crocodiles and Alligators.* Chipping Norton, Australia: Surrey Beatty and Sons Pty Ltd.
MANOLIS, S. C. & WEBB, G. J. 2016. Best Management Practices for Crocodilian Farming. *IUCN-SSC Crocodile Specialist Group, Australia*.
MARCUS, L. C. 1981. *Veterinary Biology and Medicine of Captive Amphibians and Reptiles,* Lea & Febiger.
MARZOLA, M., RUSSO, J. & MATEUS, O. 2015. Identification and comparison of modern and fossil crocodilian eggs and eggshell structures. *Historical Biology,* 27**,** 115-133.
MODHA, M. 1967. The ecology of the Nile crocodile (*Crocodylus niloticus* Laurenti) on Central Island, Lake Rudolf. *African Journal of Ecology,* 5**,** 74-95.
MORAN, E. T. 2007. Nutrition of the developing embryo and hatchling. *Poultry Science,* 86**,** 1043-1049.
MURAMATSU, T., HIRAMOTO, K., KOSHI, N., OKUMURA, J., MIYOSHI, S. & MITSUMOTO, T. 1990. Importance of albumen content in whole-body protein synthesis of the chicken embryo during incubation. *British Poultry Science,* 31**,** 101-106.
MURRAY, J. D., DEEMING, D. & FERGUSON, M. 1990. Size-dependent pigmentation-pattern formation in embryos of *Alligator mississippiensis*: time of initiation of pattern generation mechanism. *Proceedings of the Royal Society of London. B. Biological Sciences,* 239**,** 279-293.
NELSON, T. C., GROTH, K. D. & SOTHERLAND, P. R. 2010. Maternal investment and nutrient use affect phenotype of American alligator and domestic chicken hatchlings. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 157**,** 19-27.
NICOTERA, P., BELLOMO, G. & ORRENIUS, S. 1992. Calcium-mediated mechanisms in chemically induced cell death (abstract only). *Annual Review of Pharmacology and Toxicology,* 32**,** 449-470.
NOBLE, R., DEEMING, D., FERGUSON, M. & MCCARTNEY, R. 1990. Changes in the lipid and fatty acid composition of the yolk during embryonic development of the alligator (*Alligator mississipiensis*). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 96**,** 183-187.
NOBLE, R., DEEMING, D., FERGUSON, M. & MCCARTNEY, R. 1991. The utilisation of yolk phosphoglycerides by the alligator during embryonic development. *Journal of Comparative Physiology B,* 161**,** 383-386.
NOBLE, R. & MOORE, J. 1967. The partition of lipids between the yolk and yolk-sac membrane during the development of the chick embryo. *Canadian Journal of Biochemistry,* 45**,** 949-958.
NÖTHLING, J. O. 2018 (personal communication). Observations on the development of the opaque band in *Crocodylus niloticus* eggs.

NÖTHLING, J. O. 2019 (personal communication). DNA from Nile crocodile foetal membranes confirms multiple sires per clutch.
NÖTHLING, J. O., NÖTHLING, J. A. & MYBURGH, J. G. 2019a. A model by which to estimate the volume of Nile crocodile eggs after they have hatched. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie,* 38 (2019).
NÖTHLING, J. O., NÖTHLING, J. A., MYBURGH, J. G. & NÖTHLING, J. 2019b. The relationship between hatchling mass and egg volume in the Nile crocodile (*Crocodylus niloticus*): The productivity of eggs varies. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie,* 38 (2019).
NYS, Y., HINCKE, M., ARIAS, J., GARCIA-RUIZ, J. & SOLOMON, S. 1999. Avian eggshell mineralization. *Poultry and Avian Biology Reviews,* 10(3), 143–166
PACKARD, G. C., TAIGEN, T. L., PACKARD, M. J. & SHUMAN, R. D. 1979. Water-vapor conductance of testudinian and crocodilian eggs (Class Reptilia). *Respiration Physiology,* 38**,** 1-10.
PACKARD, M. J. 1994. Patterns of mobilization and deposition of calcium in embryos of oviparous, amniotic vertebrates. *Israel Journal of Ecology and Evolution,* 40**,** 481-492.
PACKARD, M. J. & CLARK, N. B. 1996. Aspects of calcium regulation in embryonic lepidosaurians and chelonians and a review of calcium regulation in embryonic archosaurians. *Physiological Zoology,* 69**,** 435-466.
PACKARD, M. J. & PACKARD, G. C. 1989. Mobilization of calcium, phosphorus, and magnesium by embryonic alligators (*Alligator mississippiensis*). *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology,* 257**,** R1541-R1547.
PACKARD, M. J., PACKARD, G. C. & GUTZKE, W. 1984. Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. *Journal of Experimental Biology,* 110**,** 99-112.
PALMER, B. & GUILLETTE, L. 1992. Alligators provide evidence for the evolution of an archosaurian mode of oviparity. *Biology of Reproduction,* 46**,** 39-47.
PETERKA, M., YVES SIRE, J., HOVORAKOVA, M., PROCHAZKA, J., FOUGEIROL, L., PETERKOVA, R. & VIRIOT, L. 2010. Prenatal development of *Crocodylus niloticus niloticus* Laurenti, 1768. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* 314**,** 353-368.
PIÑA, C. I., LARRIERA, A., MEDINA, M. & WEBB, G. J. 2007. Effects of incubation temperature on the size of *Caiman latirostris* (Crocodylia: Alligatoridae) at hatching and after one year. *Journal of Herpetology,* 41**,** 205-211.
PLATT, S. G., MONYRATH, V., SOVANNARA, H., KHENG, L. & RAINWATER, T. R. 2012. Nesting phenology and clutch characteristics of captive Siamese Crocodiles (*Crocodylus siamensis*) in Cambodia. *Zoo Biology,* 31**,** 534-545.
POOLEY, A. 1962. The Nile crocodile *Crocodylus niloticus*. Notes on the incubation period and growth rate of juveniles. *Lammergeyer,* 11**,** 1-55.
POOLEY, A. 1969. Preliminary studies on the breeding of the Nile crocodile *Crocodylus niloticus* in Zululand. *Lammergeyer,* 10**,** 22-44.
POOLEY, A. C. & GANS, C. 1976. The Nile crocodile. *Scientific American,* 234**,** 114-125.
POOLEY, T. 1982. *Discoveries of a Crocodile Man,* HarperCollins.
RATCLIFFE, D. A. 1967. Decrease in eggshell weight in certain birds of prey. *Nature,* 215**,** 208.
ROMANOFF, A. L. & ROMANOFF, A. J. 1949. The avian egg. John Wiley and Sons. New York

SADASIVAIAH, S., TOZAN, Y. & BREMAN, J. G. 2007. Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? *The American Journal of Tropical Medicine and Hygiene,* 77**,** 249-263.

SCHWABL, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences, United States of America* 90**,** 11446-11450.

SHAKER, J. L. & DEFTOS, L. 2018. Calcium and phosphate homeostasis. *Endotext [Internet]*. MDText. com, Inc.

SHILTON, C., BROWN, G., CHAMBERS, L., BENEDICT, S., DAVIS, S., AUMANN, S. & ISBERG, S. 2014. Pathology of runting in farmed saltwater crocodiles (*Crocodylus porosus*) in Australia. *Veterinary Pathology,* 51**,** 1022-1034.

SHINE, R. & BROWN, G. P. 2007. Adapting to the unpredictable: reproductive biology of vertebrates in the Australian wet–dry tropics. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 363**,** 363-373.

SHRIVASTAVA, A. 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists,* 2**,** 21.

SIMKISS, K. 1991. Fluxes during embryogenesis. *Egg incubation: its effects on embryonic development in birds and reptiles***,** 47-52.

SKAARE, J., INGEBRIGTSEN, K., AULIE, A. & KANUI, T. 1991. Organochlorines in crocodile eggs from Kenya. *Bulletin of environmental contamination and toxicology,* 47**,** 126-130.

SOLOMON, S., BAIN, M., CRANSTOUN, S. & NASCIMENTO, V. 1994. Hen's egg shell structure and function. *Microbiology of the Avian Egg.* Springer.

SOTHERLAND, P. & RAHN, H. 1987. On the composition of bird eggs. *The Condor,* 89**,** 48-65.

STATON, M. A. & DIXON, J. R. 1977. *Breeding biology of the spectacled caiman, Caiman crocodilus crocodilus, in the Venezuelan Llanos,* United States Department of the Interior, Fish and Wildlife Service.

STEWART, J. R. & ECAY, T. W. 2010. Patterns of maternal provision and embryonic mobilization of calcium in oviparous and viviparous squamate reptiles. *Herpetol Conserv Biol,* 5**,** 341-359.

STOKER, C., ZAYAS, M. A., FERREIRA, M., DURANDO, M., GALOPPO, G. H., RODRIGUEZ, H. A., REPETTI, M. R., BELDOMENICO, H. R., CALDINI, E. & LUQUE, E. H. 2013. The eggshell features and clutch viability of the broad-snouted caiman (*Caiman latirostris*) are associated with the egg burden of organochlorine compounds. *Ecotoxicology and Environmental Safety,* 98**,** 191-195.

SWANEPOEL, D. G. J., FERGUSON, N. S. & PERRIN, M. R. 2000. Nesting ecology of Nile crocodiles *(Crocodylus niloticus)* in the Olifants River, Kruger National Park. *Koedoe,* 43(2), 35–46

TARCHANOFF, J. 1884. Ueber die Verschiedenheiten des Eiereiweisses bei befledert geborenen (Nestflüchter) und bei nackt geborenen (Nesthocker) Vögeln und über die Verhältnisse zwischen dem Dotter und dem Eiereiweiss (Biologisch-chemische Untersuchung). *Pflügers Archiv European Journal of Physiology,* 33**,** 303-378.

TEREPKA, A. 1963. Structure and calcification in avian egg shell. *Experimental cell research,* 30**,** 171-182.

TEREPKA, A., COLEMAN, J., ARMBRECHT, H. & GUNTHER, T. Transcellular transport of calcium. Symp. Soc. Exp. Biol, 1976. 117-140.

THOMPSON, J., MANORE, M. & VAUGHAN, L. 2013. *Science of Nutrition, The: Pearson New International Edition,* Pearson Higher Ed.

THOMPSON, M., SPEAKE, B. & DEEMING, D. 2004. Egg morphology and composition. *Reptilian incubation: environment, evolution and behaviour***,** 45-74.
THOMPSON, M. B. 1989. Patterns of metabolism in embryonic reptiles. *Respiration physiology,* 76**,** 243-255.
THORBJARNARSON, J. 1999. Crocodile tears and skins: international trade, economic constraints, and limits to the sustainable use of crocodilians. *Conservation Biology,* 13**,** 465-470.
THORBJARNARSON, J. B. 1996. Reproductive characteristics of the order Crocodylia. *Herpetologica***,** 8-24.
THORBJARNARSON, J. B. & HERNÁNDEZ, G. 1993. Reproductive ecology of the Orinoco crocodile (*Crocodylus intermedius*) in Venezuela. I. Nesting ecology and egg and clutch relationships. *Journal of Herpetology***,** 363-370.
TRACY, R. C. & SNELL, H. L. 1985. Interrelations among water and energy relations of reptilian eggs, embryos, and hatchlings. *American Zoologist,* 25**,** 999-1008.
TUAN, R. S. 1979. Vitamin K-dependent gamma-glutamyl carboxylase activity in the chick embryonic chorioallantoic membrane. *Journal of Biological Chemistry,* 254**,** 1356-1364.
TUAN, R. S., CARSON, M. J., JOZEFIAK, J. A., KNOWLES, K. A. & SHOTWELL, B. A. 1986. Calcium-transport function of the chick embryonic chorioallantoic membrane. I. In vivo and in vitro characterization. *Journal of Cell Science,* 82**,** 73-84.
URIBE, M. C. A. & GUILLETTE, L. J. 2000. Oogenesis and ovarian histology of the American alligator Alligator mississippiensis. *Journal of Morphology,* 245**,** 225-240.
VERDADE, L. 2001. Allometry of reproduction in broad-snouted caiman (*Caiman latirostris). Brazilian Journal of Biology,* 61**,** 431-435.
WEBB, G., MANOLIS, S., BUCKWORTH, R. & SACK, G. 1983a. An examination of *Crocodylus porosus* nests in two northern Australian freshwater swamps, with an analysis of embryo mortality. *Wildlife Research,* 10**,** 571-605.
WEBB, G. & MANOLIS, S. C. Crocodile Farming — Research Priorities. Intensive tropical Animal Production Seminar, 1991 1991 Townsville Sheraton Breakwater Casino-Hotel. Queensland Department of Primary Industries, Townsville, QLD.
WEBB, G., MANOLIS, S. C. & WHITEHEAD, P. J. 1987. *Wildlife Management: Crocodiles and Alligators*, Surrey Beatty & Sons.
WEBB, G., REYNOLDS, S., BRIEN, M., MANOLIS, C. & BRIEN, J. 2013. Improving Australia's Crocodile Industry Productivity: Nutritional requirements, feed ingredients and feeding systems for farmed crocodile production.
WEBB, G. J. & COOPER-PRESTON, H. 1989. Effects of incubation temperature on crocodiles and the evolution of reptilian oviparity. *American Zoologist,* 29**,** 953-971.
WEBB, G. J., MANOLIS, S. & BUCKWORTH, R. 1983b. *Crocodylus johnstoni* in the McKinlay River Area N. T, VI.* Nesting Biology. *Wildlife Research,* 10**,** 607-637.
WEBB, G. J. & SMITH, A. M. Sex ratio and survivorship in the Australian freshwater crocodile *Crocodylus johnstoni*. Symp. Zool. Soc. Lond, 1984. 319-355.
WHITE, H. 1991. Maternal diet, maternal proteins and egg quality. *Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge***,** 1-15.
WHITEHEAD, P. 1987. Respiration of *Crocodylus johnstoni* embryos. In: Webb, G., Manolis, S. C. & Whitehead, P. J. 1987. *Wildlife management: Crocodiles and Alligators. Surrey Beatty, Sydney***,** 473-497.
WHITEHEAD, P., WEBB, G. & SEYMOUR, R. 1990. Effect of incubation temperature on development of Crocodylus johnstoni embryos. *Physiological Zoology,* 63**,** 949-964.

WHITEHEAD, P. J. 1990. Yolk Depletion and Metabolic Rate of Hatchling *Crocodylus johnstoni*. *Copeia,* 1990**,** 871-875.
WINK, C. S. & ELSEY, R. M. 1986. Changes in femoral morphology during egg-laying in *Alligator mississippiensis*. *Journal of Morphology,* 189**,** 183-188.
WINK, C. S. & ELSEY, R. M. 1994. Morphology of shells from viable and nonviable eggs of the Chinese alligator (*Alligator sinensis*). *Journal of Morphology,* 222**,** 103-110.
WINK, C. S., ELSEY, R. M. & BOUVIER, M. 1990. Porosity of eggshells from wild and captive, pen-reared alligators (*Alligator mississippiensis*). *Journal of Morphology,* 203**,** 35-39.
WU, X.-B. & HU, Y. 2010. Multiple paternity in Chinese alligator (*Alligator sinensis*) clutches during a reproductive season at Xuanzhou Nature Reserve. *Amphibia-Reptilia,* 31**,** 419-424.
YADGARY, L., YAIR, R. & UNI, Z. 2011. The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes. *Poultry Science,* 90**,** 410-416.
YOSHIZAKI, N., ITO, Y., HORI, H., SAITO, H. & IWASAWA, A. 2002. Absorption, transportation and digestion of egg white in quail embryos. *Development, growth & differentiation,* 44**,** 11-22.

Appendix A: Certificate of analysis of multivitamin CRM

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 3280

Multivitamin/Multielement Tablets

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of vitamins, carotenoids, and elements in dietary supplement tablets and similar matrices. This SRM can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3280 consists of five bottles, each containing 30 tablets. The SRM is provided as whole tablets because some of the vitamins are coated or encapsulated to provide stability and grinding would compromise this coating. Each tablet weighs approximately 1.5 g.

The development of SRM 3280 was a collaboration between the National Institute of Standards and Technology (NIST) and the National Institutes of Health (NIH), Office of Dietary Supplements (ODS).

Values were derived from the combination of results provided by NIST and collaborating laboratories. The certified and reference values in this material are the equally weighted means of the individual sets of NIST results and the means of the individual sets of measurements made by collaborating laboratories, as available. The associated uncertainties are expanded uncertainties at the 95 % level of confidence, as described below [1–4]. Values are reported on a dry-mass basis in mass fraction units [5].

Certified Mass Fraction Values: The certified mass fraction values of selected vitamins, carotenoids, and elements are provided in Tables 1 and 2. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [6].

Reference Mass Fraction Values: Reference mass fraction values for additional vitamins, carotenoids, and elements are provided in Tables 3 and 4. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [6] and are provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Certification: The certification of **SRM 3280** is valid, within the measurement uncertainty specified, until **31 October 2019**, provided the SRM is handled and stored in accordance with instructions given in this certificate (see "Warning and Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Support for the development of SRM 3280 was provided in part by the NIH-ODS. Technical consultation was provided by J.M. Betz of NIH-ODS.

Coordination of the technical measurements leading to the certification of this SRM was performed by L.C. Sander and S.A. Wise of the NIST Chemical Sciences Division and K.E. Sharpless of the Special Programs Office. Acquisition of the material was coordinated by K.E. Sharpless.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Carlos A. Gonzalez, Chief
Chemical Sciences Division

Gaithersburg, MD 20899
Certificate Issue Date: 11 May 2016
Certificate Revision History is on Page 9

Steven J. Choquette, Acting Director
Office of Reference Materials

Table 2. Certified Mass Fraction Values for Selected Elements in SRM 3280

	Mass Fraction (mg/g)			Coverage Factor, k
Boron[a,b]	0.141	±	0.007	2.36
Calcium[c,d,e]	110.7	±	5.3	2.45
Chloride[b,e]	53.0	±	2.3	2.45
Copper[b,c,e,f]	1.40	±	0.17	2.12
Iodine[a,c]	0.1327	±	0.0066	2.23
Iron[b,c,d,e]	12.35	±	0.91	3.18
Magnesium[c,d,e]	67.8	±	4.0	2.31
Manganese[c,d,e]	1.44	±	0.11	2.57
Phosphorus[d,e,f]	75.7	±	3.2	2.16
Potassium[b,d,e,f]	53.1	±	7.0	2.02
Zinc[c,e,f]	10.15	±	0.81	2.00
	Mass Fraction (µg/g)			**Coverage Factor, k**
Arsenic[a,g]	0.132	±	0.044	2.00
Chromium[c,d]	93.7	±	2.7	2.06
Lead[h]	0.2727	±	0.0024	2.14
Molybdenum[c,d,f]	70.7	±	4.5	2.57
Nickel[a,e]	8.43	±	0.30	2.00
Selenium[a,c]	17.42	±	0.45	2.00
	Mass Fraction (ng/g)			**Coverage Factor, k**
Cadmium[h]	80.15	±	0.86	2.03

[a] ICP-MS (NIST)
[b] PGAA (NIST)
[c] INAA (NIST)
[d] XRF (NIST)
[e] GMA
[f] ICP-OES (NIST)
[g] RNAA (NIST)
[h] ID ICP-MS (NIST)

Note: pages 2-6 and 8-13 of this datasheet contain safety warnings, storage instructions, legal disclaimers and data relevant to other chemical substances, and are omitted here.

Appendix B: Certificate of analysis of calcium carbonate powder

certificate of analysis

This is to certify that the below listed product has been analysed and conforms to the minimum specifications listed

CALCIUM CARBONATE powder

ace associated chemical enterprises

10 amethyst road
theta ext6
johannesburg
po box 82778
southdale 2135
tel: +27 (0)11 496 3300
fax: +27 (0)11 496 3311
www.acechem.co.za

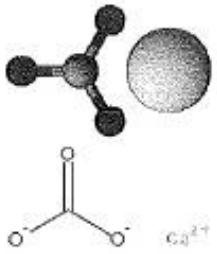
Batch No. 32898
Date 2017-03-22

GCI_2017_1/4

CC486NN Platinum Line	
Warranty certificate	
Assay	99%
Arsenic (As)	0.0003%
Chloride (Cl)	0.02%
Copper (Cu)	0.001%
Iron (Fe)	0.05%
Lead (Pb)	0.0003%
Loss on drying (200°C)	2%
Phosphate (PO_4)	0.05%
Sulphate (SO_4)	0.2%

Packaging
250g white plastic jar

Physical and chemical properties, composition and other data	
Chemical formula	$CaCO_3$ Hit: $CCaO_3$
Chemical group	Carbonates
Synonym	Carbonic acid calcium salt
Occurrence in nature	Aragonite, calcite and vaterite
Atomic weight	100.09
Appearance	White marble chips or powder
Solubility	Soluble in diluted acids
Melting point	825°C and decomposes
pH (aqueous solution)	9.5 - 9.9
Incompatible substances	Acids, alum and ammonium salts
Products of decomposition	Calcium oxide and carbon dioxide



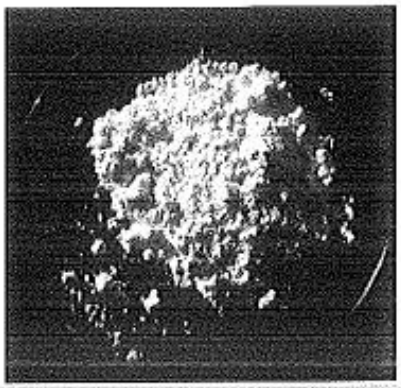
Laboratory applications and practices	
Usage	Analytical reagent. Water analysis
Filter paper	Filtech no: 0225, 0282, 1803, 1830

Storage and handling information	
Storage	Store separated from incompatible substances
Colour code	Green
Disposal	3

Transport regulations	
Tariff number	2836.50.00

Identifiers	
Cas number	471-34-1
EC/EINECS number	207-439-9
RTECS number	FF9335000

Exp 11/9/2021



QUALITY WITHOUT COMPROMISE

Appendix C: Certificate of analysis of potassium hydrogen phosphate powder

certificate of analysis

This is to certify that the below listed product has been analysed and conforms to the minimum specifications listed

di-POTASSIUM HYDROGEN ORTHOPHOSHATE

ace associated chemical enterprises

10 amethyst road
theta ext6
johannesburg
po box 82778
southdale 2135
tel: +27 (0)11 496 3300
fax: +27 (0)11 496 3311
www.acechem.co.za

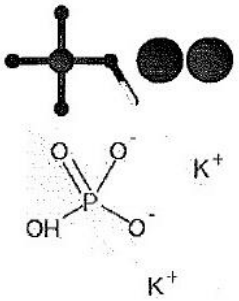
Batch No. 27204
Date 2017-03-22

GD4-2017-1/3

P2060NN	Gold Line
Typical analysis	
Assay	min 98%
Arsenic (As)	0.0002%
Cadmium (Cd)	0.0001%
Chloride (Cl)	0.01%
Fluorine (F)	0.001%
Iron (Fe)	0.005%
Loss on drying (105°)	max 2%
Lead (Pb)	0.0005%
Mercury (Hg)	0.0001%
Sulphate (SO_4)	0.02%
Water-insoluble matter	0.05%

Packaging
500g white plastic jar

Physical and chemical properties, composition and other data	
Chemical formula	K_2HPO_4 Hill: HK_2O_4P
Synonyms	di-Potassium phosphate secondary; Potassium biphosphate; Potassium monophosphate; DKPPotassium phosphate di-basic; Secondary potassium phosphate
Chemical group	Phosphates
Atomic weight	174.18
Appearance	Small white hygroscopic crystals or very fine powder
Solubility	Water
Density (g/ml)	2.44
pH (aqueous solution)	9.1



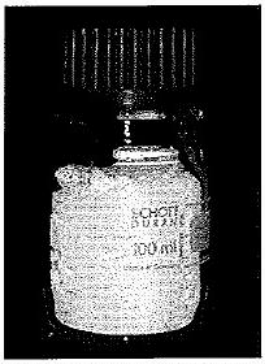
Laboratory applications and practices	
Usage	Analytical reagent

Storage and handling information	
Storage	Keep container tightly closed
Colour code	Green
Disposal	3

Transport regulations	
Tariff code	2835.24.00

Identifiers	
Cas number	7758-11-4
EC/EINECS number	231-834-5
RTECS number	TC5580000

Exp 23/2/2025



QUALITYWITHOUTCOMPROMISE

Form ref: SCA03 Effective date: 15/03/08

Appendix D: Calibration certificate for microwave digester

Cert 019_2017

MAD TECHNOLOGY

CERTIFICATE OF CALIBRATION

M.A.D TECHNOLOGY (PTY) LTD hereby certifies that of this date the CEM instrumentation, as specified below, meets or exceeds all published specifications and has been calibrated using standards traceable to the national Institution of Standards and Technology (NIST). Records pertaining to these standards are on file and are available for examination.

INSTRUMENT MODEL: **CEM MARS 6 MICROWAVE DIGESTER**

INSTRUMENT SERIAL NUMBER: **MJ 6039**

DATE OF CALIBRATION: **09 MAY 2017** EXPIRATION DATE: **15 MAY 2018**

CALIBRATION DATA

ITEM	ACTUAL READING	TOLERENCE
INNER SENSOR	130°C	130°C ± 2°C
OUTER SENSOR	130°C	130°C ± 2°C
POWER 800 WATT	878.9	712 WATT
POWER 1000 WATT	1029.3	890 WATT
Power 1800 WATT	1842.4	1600 WATT
PRESSURE	200 PSI	200 PSI +/- 10 PSI

CALIBRATION EQUIPMENT USED

ITEM	SERIAL NUMBER	VALIDITY
Hanna Checktemp	054ADF	JUNE 2017
INTELLITEMP 4014	A92500	NOVEMBER 2017
BITS DIAGNOSTIC IN TER	CP 1068	INST CAL VALUES

MAD TECHNOLOGY ASSOCIATE

M.A.D TECHNOLOGY (PTY) LTD P.O BOX 4040, RIVONIA, 2128, SOUTH AFRICA,

UNIT G1G PINEWOOD SQUARE, PINEWOOD OFFICE PARK, 33 RILEY ROAD, JOHANNESBURG, SOUTH AFRICA

TEL +27 11 797 3600, FAX +27 11 797 3675, EMAIL bruce@madtech.co.za

Appendix E: Calibration certificate for microbalance

Cert 029 - 2017

CALIBRATION CERTIFICATE — Page 1 of 1

SARTO MASS SERVICES cc
Balance Services and Calibration

Reg No. CK 2001/040589/23
328 Boundary Road, North Riding, 2169
P O Box 15, Randburg 2125
Tel: (011) 787-9178
Fax: (086) 609 1369

sanas Calibration Laboratory
1415

Customer Name:	Nutritek Laboratories	Location:	Weighing Organics	Amended Cert:	No
		Environment:	Dusty	Original Cert No.:	JVZ 10948
Customer address where calibration was performed:	Nutro Business Hub	Next Due:	Jan-18	Job card no:	13278
	9 de Ou Wapad Road	Calibration date:	21-Jul-17		
	Hartebeespoort	Certificate issue date:	31-Jul-17		
	0250	Metrologist:	J. Van Zyl	Procedure(s) Used:	
Contact Person:	George Johnson	Checked by:	T. Maseng / C. Lottering	SMS001/CP	

Standards Used		Description of UUT	
Description 1	Standard Set of Massplaces	Make / Model :	PRECISA / XT220A
Certificate no.	M16-220 did Mar-2016	Serial No. :	2801755
Description 2	Standard Set of Massplaces	Unit of Measurement:	g
Certificate no.	M16-422 did Jun-2016	Capacity:	220
Description 3	Standard Set of Massplaces	Resolution:	0.0001
Certificate no.			

CALIBRATION CERTIFICATE: This calibration certificate is issued under the authority and conditions granted by SANAS. It may not be reproduced except in full, without the written approval of the laboratory.
CALIBRATION PROCEDURE: The instrument has been calibrated with standard weights of known value, traceable to the national standard, in accordance with Procedure No. SMS001/CP.
COMMENTS: SETTLING TIME 20 SECONDS. INTERNAL CALIBRATION DONE.

		ACCURACY AND LINEARITY			
REPEATABILITY	LOAD APPLIED	BEFORE	AFTER	AFTER ADJ. DEV.	UNIT USED
99.9998	0.1000	0.1000	0.1003	0.0003	g
99.9998	0.4999	0.5000	0.5003	0.0004	
99.9998	0.9999	1.0000	1.0004	0.0005	
99.9998	2.0000	2.0001	2.0004	0.0004	
99.9998	5.0000	5.0001	5.0002	0.0002	
99.9999	9.9999	10.0000	10.0000	0.0001	
Std Deviation Unit Used:	19.9998	19.9999	19.9997	-0.0001	
g	49.9996	49.9999	49.9998	0.0002	
Std Deviation:	99.9995	100.0001	99.9998	0.0003	
0.0040	219.9950	219.9960	219.9951	0.0001	

Eccentric Error

Unit Used:	Load Applied:		Error:	Unit Used:
g	50.0000		0.0006	g
		50.0004		
	50.0002	49.9998	50.0001	
		49.9998		

EXPANDED UNCERTAINTY (u): 0.0004 g

EXPANDED UNCERTAINTY: The reported expanded uncertainty is based on a standard uncertainty multiplied by a coverage factor k=2 providing a level of confidence of approximately 95%, the uncertainty of measurement has been estimated in accordance with the principles defined in the GUM, Guide to Uncertainty of Measurement, ISO, Geneva, 1993'.
VALIDITY OF CALIBRATION: The measurement results recorded in this certificate were correct at the time of calibration. The subsequent accuracy will depend on factors such as care, handling and frequency of use. It is recommended that the recalibration be undertaken at an interval that will ensure that the instrument remains within the declared limits.

Calibrated By:
J. Van Zyl
Technician

Checked By:
Quality Manager /
Technical Manager

Authorised By:
C. Muirhead
Technical Signatory

End of Calibration Certificate

Appendix F: Animal ethics committee approval certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**A descriptive study of the variance in calcium concentration and total calcium within infertile eggs of farmed Nile crocodiles *(Crocodylus niloticus)***
PROJECT NUMBER	**V109-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. GJ Brown**

STUDENT NUMBER (where applicable)	**UP_02392135**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Eggs**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**December 2015-December 2016**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	30 November 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

S4285-15

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**The movement of calcium from the shell, shell membrane, yolk and albumen to the developing foetus of the Nile crocodile (*Crocodylus niloticus* Laurenti)**
PROJECT NUMBER	**V104-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. GJ Brown**

STUDENT NUMBER (where applicable)	**UP_24029549**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Eggs: *Crocodylus niloticus***	
NUMBER OF ANIMALS	**30**	
Approval period to use animals for research/testing purposes		**January 2017-January 2018**
SUPERVISOR	**Prof. JO Nothling**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 January 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15