A comparison of calcium and phosphorus in components of fertile and size-matched unbanded Nile crocodile eggs

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ABSTRACT

Research in other species suggests that the source of embryonic calcium (Ca) and phosphorus (P) for *Crocodylus niloticus* is likely 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 prior to anticipated hatching, and compared with those of size-matched unbanded eggs (eggs that failed to form an opaque band around the lesser circumference, indicative of presumed infertility). Shell contained the highest Ca concentration and content, followed by the foetus, followed by the intra-abdominal yolk. Foetal tissue had the highest P concentration and content, followed by intra-abdominal yolk. The Ca and P concentration of intra-abdominal yolk of foetuses in fertile eggs varied more widely than did the yolk of unbanded eggs, based on coefficient of variation. Ca concentration of fertile egg yolk was in some cases found to exceed that of the yolk of unbanded eggs, suggesting that Ca is stored there after being removed from the shell, however, yolk Ca content was consistently lower in fertile than in unbanded 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. The Nile crocodile appears to follow the classic archosaurian pattern of Ca mobilisation, whereby the shell supplies the majority of foetal Ca, but the intra-abdominal yolk contains substantial Ca reserves for use by the hatchling. This study provides clinicians and researchers with information on sample collection and analysis of Nile crocodile egg and foetal tissue, provides baseline descriptive data on Ca and P concentration and content, discusses the effect of potential covariates on Ca and P concentration and content, and discusses the movement of Ca and P from reserves within the egg to the developing foetus.

KEYWORDS: albumen, embryo, foetus, reptile, shell, yolk

Introduction

Calcium (Ca) is essential for many physiological functions: amongst others nerve conduction, intracellular signalling, blood coagulation, as an enzyme cofactor (Veum et al. 2010), as well as mineralisation of bone and teeth (Carafoli 1987). Phosphorus (P), *inter alia,* is required for bone formation, is incorporated into cell membrane phosphoproteins, is a critical constituent of nucleic acids and adenosine triphosphate (ATP), and functions in maintaining intracellular fluid balance (Thompson et al. 2013; Shaker and Deftos 2018).

During embryonic development in oviparous species, Ca from extra-embryonic sources (yolk or shell) is moved across the chorioallantoic membrane and yolk sac membrane to the conceptus (Packard and Clark 1996). In lizards and snakes, most foetal Ca comes from the yolk (Packard et al. 1984; Simkiss 1991), but in crocodilians the situation is different: by successively opening eggs through the incubation period and analysing the Ca and P content of its compartments, Packard and Packard (1989) showed that the shell of *Alligator 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 the fertile crocodilian egg, development of the chorioallantois in early incubation results in externally visible physical changes to the shell membrane. This process is known as 'banding': failure of banding may occur due either to lack of fertilisation or early death of the embryo. Unbanded eggs are freely available, and in the present research have been used as experimental proxies for very early-stage fertile eggs under the assumption that they reflect the Ca and P content and distribution of fertile eggs prior to the onset of embryonic metabolic activity.

Issues with provision or metabolism of Ca or P can induce clinical skeletal disorders among reptiles (Marcus 1981). In captive crocodilians, glassy teeth, rubbery jaws and an increased incidence of fractures have been reported (Lane et al. 1984; Huchzermeyer 2003; Manolis and Webb 2016). Shilton et al. (2014) reported that mild hypocalcaemia, moderate hypophosphataemia, hypoalbuminaemia and osteoporosis occurred more commonly in runt *Crocodylus porosus* hatchlings than in normal-sized hatchlings. The exact role of Ca and P, if any, in the pathogenesis of runted hatchlings has not yet been established, and the role of abnormal *in ovo* metabolism of these elements on post-hatching growth and health in farmed crocodiles has not been formally studied. An improved understanding of fertile Nile crocodile egg Ca and P metabolism may be useful in diagnosing and treating clinical deficiencies manifesting as unhealthy hatchlings, as well as planning interventional studies that investigate specific effects of deficiencies or supplementation on hatchling health. Prior to any such studies, it is essential to establish baseline measurements of sample variability, evaluate the influence of predictor variables, and identify potential pitfalls in sample selection, processing and analysis, which may result in inconclusive or unhelpful results. With these broad aims in mind, the specific purposes of the present research were:

1. To describe the concentration and content of Ca and P in the compartments (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 compartments.
3. To determine the principal sources of Ca and P for the developing conceptus by comparing measurements of Ca and P concentrations and content of fertile eggs to those found in unbanded eggs of similar mass.

Materials and methods

Specimen collection and preparation

All experimental eggs originated from the same commercial farm, but from different breeding ponds during the same breeding season. Unbanded eggs and some data used in this study were also used for a prior study that measured the concentration and content of Ca and P in unbanded eggs (Brown et al. 2020). Fertile *C. niloticus* eggs ($n = 30$; one from each of 30 clutches) were collected five to ten days prior to predicted hatching date, uniquely numbered and placed on ice. Eggs were refrigerated for 24 h at 4 °C before each egg's surface was cleaned with deionised water, dried for at least an hour at room temperature, and weighed in g to three decimals. The shell at one pole of the egg was broken and shell fragments kept for later weighing, resulting in a shell-free region of approximately 30 mm 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. Although foetuses were subjected to at least 24 hours of refrigeration, upon removal of each foetus from the egg, its death was ensured according to IUCN-CSG recommended practices (Manolis and Webb 2016). The intra-abdominal yolk was removed from the abdomen of the foetus. Remaining foetal fluids, remnant albumen and foetal membranes were removed from the empty egg and placed in a weighing container. The shell was manually separated from the shell membrane. Compartments of the fertile egg (shell, shell membrane, yolk, foetus, and a mixture of foetal membranes, foetal fluids and remnant albumen) were weighed separately in preweighed containers. 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, since it was found by experimentation that freeze-drying these components rendered a more homogeneously dried, powdered product than simply oven-drying them. After freeze drying for at least 24 hours (−50°C, 80 mTorr, Air and Vacuum Technologies, Midrand, South Africa), samples were pulverised and further oven dried for approximately two hours at 50°C to constant mass, to ensure total desiccation. Shell and shell membrane samples were not freeze dried, but were oven dried for at least six hours at 50°C.

Specimen Ca and P determination

Each compartment of the 30 fertile eggs (shell, shell membrane, yolk, foetus, and a mixture of foetal membranes and residual foetal fluids) had Ca and P concentration determined using inductively coupled plasma optical emission spectroscopy (ICP-OES). Process details, method validation and limits of detection and quantification were identical to those reported in a prior study into Ca and P within unbanded eggs (Brown et al. 2020). Limit of detection for Ca was 0.01 g kg^{-1} and limit of detection for P was 0.002 g kg^{-1}. Ca and P content were calculated by multiplying dry compartment mass by element concentration. Within-sample laboratory method repeatability, as well as specimen homogeneity, was assessed by analysing six blinded duplicate aliquots, derived from three samples of each fertile egg component type.

Matching of unbanded and fertile eggs

The Ca and P concentration and content of the components of unbanded Nile crocodile eggs has been reported previously (Brown et al. 2020). Some data from that study were used for comparison with fertile egg component Ca and P data in the present study. Each fertile egg

was matched to an unbanded egg of which the mass was as close as possible to, but within five g above or below, its own mass.

Cost constraints precluded the analysis of an extremely large number of samples, it was therefore necessary to be selective. Brown et al. (2020) found that the coefficient of variation for Ca concentration in the shell of unbanded eggs was low (0.97%), and accordingly the mean Ca concentration of a randomly selected unbanded eggshell is a fair approximation of that of any other. In line with this finding and to save costs, fewer shell samples were analysed, to allow resources to be directed to analysis of more samples from other components. An effect of this strategy was that only three size-matched unbanded eggs were available that had Ca concentration data available for shell, as well as the other egg components. However, it was still possible to obtain an estimate of unbanded shell Ca content because it was found that the principle determinant of Ca content in an eggshell was the mass of that eggshell (Brown et al. 2020). The dry shell mass was therefore multiplied by the mean unbanded shell Ca concentration value (391.21 g kg^{-1}, $n = 15$, as per Brown et al. (2020)) to provide an estimate of the shell Ca content of a given unbanded egg.

When selecting matched pairs, the only criterion considered was egg mass; other factors, such as clutch, laying date and maternal pond, were not considered. For unbanded eggs, separation of shell membrane from shell was performed on dried samples. Separation of shell and shell membrane of unbanded eggs prior to drying was difficult (Brown et al. 2020, also noted by Ferguson 1982 for *Alligator mississippiensis*), therefore the wet mass of unbanded shell and shell membrane was estimated from dry mass by assuming that they contained the same mean dry fraction as those of fertile eggs, for which both wet and dry masses were explicitly measured (dry fraction of shell 96%, standard deviation 0.003, $n = 30$; dry fraction of shell membrane 45%, standard deviation 0.006, $n = 30$).

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.

Ca and P concentration and content for each compartment were graphed using histograms and inspected for normality of distribution. The Stata command ‘sktest’ was used to formally assess normality of distribution at the 5% significance level. For normally distributed concentration and content variables, the mean, standard deviation, minimum and maximum values were reported. For non-normally distributed concentration and content variables, the median, 25th and 75th percentiles, as well as minimum and maximum values were reported.

Ca and P concentration were determined on a dry matter basis, however total dry mass data of 14 of 30 unbanded yolk and albumen samples was not recorded. This missing dry mass data meant that comparison of the total amount of fertile and unbanded yolk and albumen Ca and P was confined to the remaining 16 pairs.

Where unbanded and fertile compartment Ca and P concentration and content were normally distributed, their relationship with the potential predictor variables fertility status, egg mass, pond of origin, date of laying, and clutch was assessed using a mixed-effects multilevel regression model. Size-matched pair was a second-level (grouping) variable.

To assess the association between foetal Ca (or P) content and that of the fertile egg's shell, shell membrane and yolk, a multivariable 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.

Results

Duplicate shell and shell membrane samples of six specimens of each sample type showed the lowest coefficient of variation in Ca and P concentration, whereas foetal fluid and membranes showed the highest variation (Table 1).

Table 1. Summary of mean of intra-assay coefficients of variation, expressed as percentages, of six duplicate measurements of Ca and P concentration in each fertile egg

	Ca	P
Shell	0.2	–[a]
Shell membrane	1.6	4.4
Yolk	4.7	3.4
Foetus	3.4	2.5
Foetal fluids and membranes	18.8	15.8

[a]P was below detection limit (0.002 g kg−1) in all shell samples.

Masses of the compartments of size-matched unbanded and fertile eggs

Mean masses of size-matched unbanded and fertile egg compartments are shown in Table 2. 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)

Table 2. Wet masses (g) of compartments 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.

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

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

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

Descriptive statistics for unbanded and fertile Ca and P concentrations by compartment are shown in Table 3, and those describing Ca and P content by compartment are shown in Table 4. All concentrations are expressed on a dry matter basis.

Table 3. Concentrations of Ca and P (in g kg^{-1} of dry matter) in the compartments 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.

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

Table 4. Descriptive statistics for Ca and P content (in g) 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			0.03	0.01	0.05	0.02	0.004				29
Yolk	0.23	0.04				0.05	0.02				16
Albumen	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^{-1}.

As had been shown previously for unbanded eggs (Brown et al. 2020), fertile eggshell Ca concentration had low variability between samples. Shell membrane Ca concentration was normally distributed among fertile egg samples, in contrast to the highly positively skewed data obtained for unbanded egg samples. Foetal tissue contained a substantial concentration of Ca and P relative to all other components (except shell Ca), with comparatively little variability between samples. The mixture of foetal membranes, foetal fluid and residual albumen contained a concentration of Ca and P that varied considerably, compared with foetal tissue. It is evident from Table 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.

Total egg Ca content

Mean content of Ca in fertile eggs was 0.23 g higher than that in unbanded eggs, whereas the mean content of P in fertile eggs was 0.09 g higher than that in unbanded eggs (Table 4). However, if the effect of potential confounding factors was analysed 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, $z = 3.56$, $p < 0.001$). (Wald's χ^2: 55.48, with $p < 0.001$ and DF = 8).

Fertility status was the only factor that influenced total egg P content. Fertile eggs contained a mean of 0.08 g more P than unbanded eggs (SE = 0.036, 95% CI = 0.015–0.15, $z = 2.37$, $p < 0.05$). (Wald's χ^2: 69.87, with $p < 0.001$ and DF = 8).

Shell Ca concentration and content

Shell Ca concentration was formally measured for only three unbanded eggs of 30 pairs, so formal direct comparison between size-matched unbanded and fertile shell concentration cannot be made using the current dataset. Without considering the effect of covariates, the mean measured 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 (Figure 1). However, in the multilevel regression model, egg mass was the only formally significant predictor of shell Ca content at the 0.05 level: 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, $z = 6.47$, $p < 0.001$). However, fertility status approached significance ($p = 0.075$) (Wald's χ^2: 81.51, with $p < 0.001$, and DF = 8), which hints at statistical support for the relationship apparent in Figure 1.

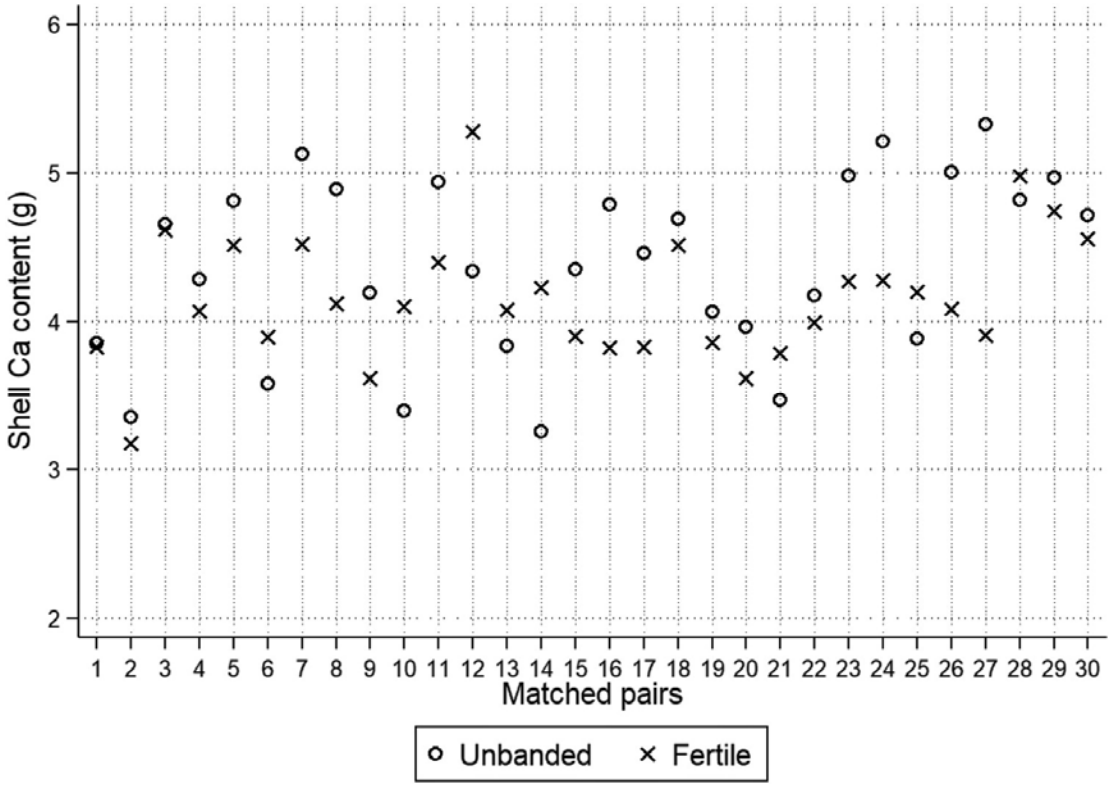

Figure 1. Difference in measured shell Ca content of fertile eggs and estimated shell Ca content of size-matched unbanded eggs.

Shell membrane Ca and P concentration and content

Shell membrane of unbanded eggs had relatively low Ca and P concentrations compared with other compartments, and a relatively large variation (% CV) in these values. Furthermore, wet mass of shell membrane of unbanded eggs was estimated from measurement of fertile eggshell membrane. Consequently, the authors are hesitant to interpret differences in Ca and P content between unbanded and fertile shell membranes from the current data.

Yolk Ca and P concentration and content

The distribution of yolk Ca and P concentrations in specimens of fertile eggs was noticeably wider than from those of corresponding size-matched unbanded eggs (Figure 2).

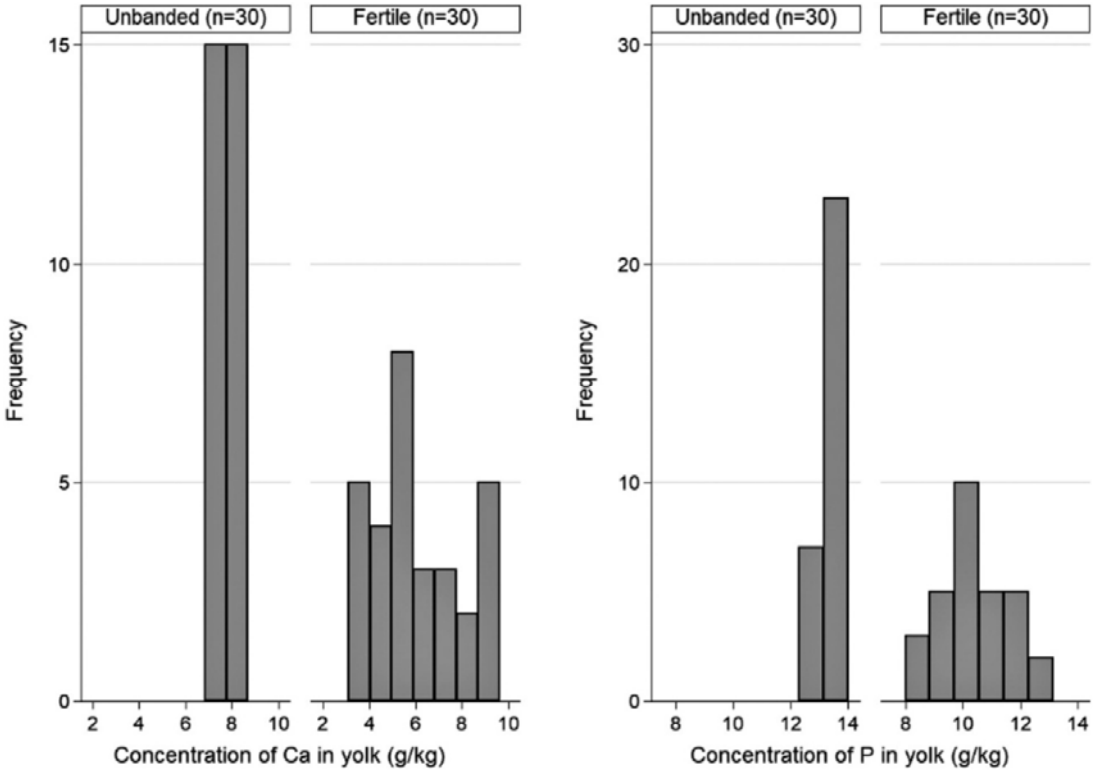

Figure 2. Frequency distribution of yolk Ca and P concentration (g kg−1 of dry matter) in unbanded and size-matched fertile eggs.

In the mixed-effects regression model, yolk Ca concentration of fertile eggs was a mean of 1.68 g kg^{-1} lower than that of unbanded eggs of similar size (SE = 0.64, 95% CI = −2.93 to −0.42, $z = -2.62$, $p < 0.05$). (Wald's χ^2 value: 35.41, with $p < 0.001$ and DF = 8). If a multilevel mixed effects model was not used and instead the mean value of fertile yolk Ca concentration (6.02 g kg^{-1}) was simply subtracted from the mean value of unbanded yolk Ca concentration (7.72 g kg^{-1}), a very similar value of 1.70 g kg^{-1} 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 a mean of 3.56 g kg^{-1} lower than that of unbanded eggs of similar size (SE = 0.41, 95% CI = −4.36 to −2.76, $z = -8.69$, $p < 0.001$). (Wald's χ^2 value: 252.80 with $p < 0.001$ and DF = 8). Once again, if the mean value of fertile yolk P concentration (10.39 g kg^{-1}) was subtracted from the mean value of unbanded yolk P concentration (13.45 g kg^{-1}), a similar value of 3.06 g kg^{-1} 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 a mean of 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, $z = -11.50$, $p < 0.001$) (Wald's χ^2 value: 1 587.34, with $p < 0.001$ and DF = 9). If 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 value of 0.18 g was obtained, a value very similar to that found using the mixed-effect regression model (Figure 3).

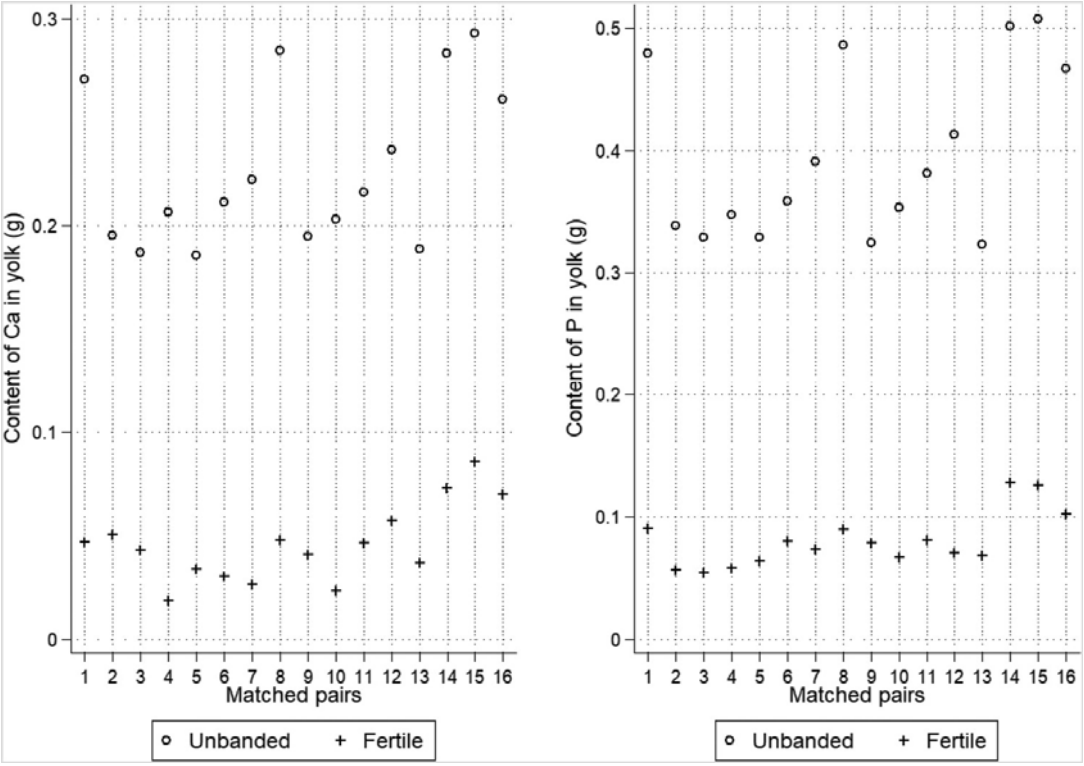

Figure 3. Comparison of Ca and P content between size-matched pairs of fertile and unbanded egg yolks.

Similarly, for yolk P content, fertile eggs had a mean of 0.26 g less P than unbanded eggs (SE = 0.03, 95% CI = −0.33 to −0.2, $z = -8.27$, $p < 0.001$) (Wald's χ^2 value: 1 401.45, 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 value of 0.32 g was obtained (Figure 3).

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

A multivariable regression model showed 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 could be 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, $r^2 = 0.15$, $p < 0.05$).

Discussion

As was seen for unbanded eggs, the shell of the fertile crocodile egg yielded the most repeatable within-sample measurements, reflecting its relatively homogeneous, largely inorganic composition, when compared with other egg components. When the mean shell Ca concentration of 15 unbanded eggs from a prior study (Brown et al. 2020) was compared with mean shell Ca concentration of fertile eggs in the present study, there was very little apparent difference (unbanded eggs: mean 391.87 g kg^{-1}, SD 3.90, $n = 15$; fertile eggs: mean 390.01 g kg^{-1}, SD 2.88, $n = 30$). When comparing measured shell Ca content of fertile eggs with the estimated shell Ca content of unbanded eggs, the only predictor of total shell Ca at the $\alpha = 0.05$ level of significance was egg mass. However, on visual evaluation of Figure 1, it is evident that within 22 of 30 size-matched pairs, unbanded eggshell contained a greater total amount of Ca than fertile eggshell, and fertility status did appear to exert an effect that approached formal statistical significance ($p = 0.075$). This finding is borne out by empirical observation: shells of fertile eggs are noticeably thinner and more easily broken than those of unbanded eggs. As in other archosaur species, Nile crocodile shell Ca is probably removed by the chorioallantois via enzymatic digestion of the innermost shell layers, resulting in a fertile eggshell of similar or identical chemical composition to that of the unbanded shell, but lighter in mass (Tuan et al. 1986).

There was lower within-sample variability for fertile eggshell membrane Ca, than was found in a prior study of unbanded eggshell membrane (Brown et al. 2020). This difference may reflect the comparative ease with which fertile egg shell membrane could be separated from the shell, due to weakening of the attachment between the organic shell membrane and mammillary layers of the shell during incubation (Ferguson 1982), thereby avoiding Ca contamination of shell membrane by shell, which was likely the source of the largest variability noted. 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 3), however total content of both elements was very low (Table 4). Because 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 Ca found in the fertile eggshell membrane represents Ca 'in transit' from shell to chorioallantois. It is unfortunate that reliable measurements of Ca (uncontaminated by shell) could not be obtained from size-matched unbanded eggshell membrane to allow for direct comparison.

Duplicate analyses of samples from the same fertile egg yolk varied more than those similarly taken from unbanded egg yolks (Table 1), which suggests an uneven utilisation of yolk Ca and P by the foetus. For Ca, one explanation may be that the portion of yolk immediately beneath the yolk sac membrane has a higher Ca concentration than the remainder of the yolk, due to active local deposition of shell-derived Ca. In *Alligator mississippiensis,* it was found that there is deposition of calcium in excess of requirements of bone mineralisation (Packard and Packard 1989): after incorporation of the yolk into the abdominal cavity prior to hatching, this extra Ca likely provides a reserve store for the neonate to use during the early posthatching period: this is the typical pattern of deposition found in crocodilians and birds, and contrasts with the pattern seen in lepidosaurians and chelonians where very little residual yolk Ca and P remains at hatching (Packard and Packard 1991; Packard 1994). It might therefore be suggested that analysis of Ca and P in the intra-abdominal yolk of dead or sacrificed sample animals may be helpful in the diagnosis of deficiency or metabolic abnormality at the farm level that may be indicative of maternal dietary deficiency, disease or suboptimal incubation conditions.

The developing foetus and recently hatched neonate benefit from a store of energy-rich yolk phosphoglycerides (Noble et al. 1991). Rather than having P uniformly bound up in such compounds and distributed evenly through the yolk, the within-sample variability in yolk P concentration suggests an uneven distribution. Movement of yolk lipid may occur between yolk and perivitelline membrane during incubation (Noble and Moore 1967; Noble et al. 1990), which may explain some variability in P concentration from different samples derived from the same yolk. Another possible explanation for the variable yolk Ca and P concentration, is simple density-based stratification: those compounds with higher density gravitate towards the more dependent portions of the yolk during incubation, a process that may be facilitated by the fact that, unlike birds' eggs, crocodile eggs are not turned continuously by the parent animal throughout incubation.

Figure 2 shows a wider distribution of Ca and P concentration in fertile egg yolks than in unbanded egg yolks. For fertile yolk Ca, a cursory inspection of the histogram in Figure 2 shows that roughly as many fertile egg yolks displayed a Ca concentration of 3–4 g kg^{-1} as those that displayed a concentration of 9–10 g kg^{-1}. Although this may be reflective of a within-sample variability in yolk Ca concentration, it is possible that such a distribution pattern indicates a snapshot of a dynamic absorption process, whereby uptake of Ca by the foetus from the yolk is followed by uptake of Ca from the shell by the chorioallantoic membrane by a vitamin K-dependent transport process as described in chickens by Akins and Tuan (1993). Alternatively, these findings could be mirroring those of Packard and Packard (1989), who 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 compartment 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, there was some between-sample variability in the time between projected hatching and the date of sample collection, so the spread in Ca concentration may reflect different stages of the prehatching uptake of Ca by the yolk. In the formal 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 relatively limited sample size and a relatively small sampling window of approximately 5 days, which may have been too short a period to identify a difference.

An alternative explanation for the shape of the graphs for fertile egg Ca and P seen in Figure 2, is that there is a continuous two-way 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 3 shows that there is clearly far less total Ca and P in fertile yolks in late incubation, than in size-matched unbanded yolks, indicating that the yolk is an important source of these elements for the developing foetus: an unsurprising finding, given the large body of prior research in other species.

In contrast to Ca and P content, the Ca concentration of fertile egg yolk was approximately the same or higher than that of size-matched unbanded egg yolk, whereas P concentration in the yolk of fertile eggs was lowers than in the yolk of size-matched unbanded eggs. Again, this emphasises that there is a source of Ca outside of the yolk in the shell, whereas there is no such supplementary source of P.

Compared with 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 compartment contributed little to foetal Ca and P.

In selecting an unbanded egg to pair with each fertile egg, only the relative masses of the two eggs were considered. Other possible covariates, such as clutch (female) of origin, breeding pond, or time within the laying season, were not considered: instead these potential sources of variability were accounted for in statistical models. In support of such an approach, it has been shown previously that eggs of similar mass may be expected to have yolks of similar mass (Brown et al. 2019) with similar Ca and P content (Brown et al. 2020). An alternative approach may have been to remove an egg of unknown fertility status from a clutch immediately after laying, arrest its development by chilling, and use it for direct within-clutch comparison with an egg near the point of hatching. If purchase of a larger number of fertile eggs is possible, this approach may be easier to implement prospectively, since eggs are more similar in mass within than between clutches (Brown et al. 2019). This approach would obviate the need to weigh many randomly selected unbanded eggs to find an optimally size-matched pair, and simplify statistical analysis by removing clutch-associated confounders. Such a method has been applied in earlier reptile egg research and it lends itself most easily to the study of eggs from wild nests (Packard et al. 1984; Packard and Packard 1989); however, it has the disadvantage of increased cost and potential loss of income to the commercial farmer.

The present research describes Ca and P concentration and content in the compartments 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, a finding similar to that made by Packard and Packard (1989) in *A. mississippiensis*. Despite the drawbacks mentioned, the present study provides details on sample collection and processing, demonstrates potential pitfalls in sample collection and analysis, and provides baseline data on Ca and P concentration and content in fertile eggs of the Nile crocodile that may be used for planning of future studies and in the investigation of disorders associated with Ca or P metabolism at the farm level.

Acknowledgements

We thank Mr Stefan van As and Ms Karon Griffiths for provisioning of sample materials and assistance during data collection, as well as Mr Kgomotso Sekwele for assistance in egg processing.

Ethics statement

The Animal Ethics committee of the University of Pretoria approved all procedures (certificates v104-16 and v109-15).

Funding

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

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