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

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ABSTRACT

Unbanded crocodilian eggs do not form an opaque band around their lesser circumference, indicating fertilization failure or early embryonic death. Assuming they represent fertile eggs prior to the onset of embryonic metabolism, the concentration and content of calcium (Ca) and phosphorus (P) in each component (shell, shell membrane, yolk and albumen) of unbanded farm-laid Nile crocodile (*Crocodylus niloticus*) eggs were described. The grouping effect of clutch (clutch effect) on each component's Ca and P concentration and content were assessed. Using regression models, the clutch size, clutch laying date, pond of origin, component mass and component Ca and P concentration were evaluated for an effect on each component's Ca and P content. Eggshell made by far the greatest contribution to total egg Ca whilst contributing no measurable P. Yolk contributed by far the greatest quantity of P and a significant quantity of Ca. Albumen contributed variable, but generally very low quantities of Ca and P to the egg. A strong clutch effect existed for shell Ca content and yolk Ca and P concentration and content. A very weak clutch effect existed for shell Ca concentration, and albumen Ca and P concentration. Shell membrane was an unreliable sample type in this study, likely reflecting issues with processing. Shell Ca and yolk Ca and P content were influenced primarily by component mass, and secondarily by element concentration. Albumen Ca and P content was principally influenced by element concentration. These descriptive findings will guide sample selection for future research.

Keywords: albumen; clutch; crocodilian; element; shell; shell membrane; yolk

1 INTRODUCTION

Crocodilians are grown for their skin and meat in subtropical and tropical regions worldwide (Thorbjarnarson, 1999). In Southern Africa, the indigenous Nile crocodile (*Crocodylus niloticus*) is farmed intensively (Caldwell, 2017; Carruthers, 2008; Fergusson, 2010). Hatchlings grown to slaughter are produced from artificially incubated eggs, which are either harvested from wild nests or laid by captive females (Thorbjarnarson, 1999). Amongst crocodilians, the clutch size and hatching rates of eggs from wild nests may differ from those laid in captivity: Joanen and McNease (1977) found that *Alligator mississippiensis* eggs from wild nests had higher hatching rates than those laid by captive females. Khosa, Imbayarwo-Chikosi, and Hamandishe (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. Regional variation in mean female age or size was not assessed by Khosa et al. (2012), although Thorbjarnarson (1999) found these metrics to correlate positively with clutch size. Whilst female factors such as age, size or genotype may influence clutch size, egg size, egg composition and hatching rate, it is also likely that environmental influences such as food availability, dietary composition, habitat type and climate may play a role.

In captivity, where the physiological, dietary and environmental requirements of the animal are entirely at the mercy of farm management, it is likely that differences in feeding and husbandry may affect embryo and hatchling health. For example, Isberg, Thomson, Nicholas, Barker, and Moran (2005) found that *Crocodylus porosus* females kept in larger pens had larger average clutch sizes and produced a higher proportion of viable hatchlings than those kept in smaller pens.

Lance, Joanen, and McNease (1983) suggested that maternal dietary micro- or macronutrient or fatty acid composition may influence alligator egg hatching rate. Inadequate provision or pathological metabolism of calcium (Ca) and phosphorus (P) is common in captive reptiles (Marcus, 1981). Symptoms of Ca- and P-associated metabolic bone disease in crocodile hatchlings were 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.

Embryos of birds and oviparous reptiles obtain Ca from yolk and eggshell (Simkiss, 1991). The process of mobilization of shell Ca during incubation in crocodilians results in thinning of the shell (Soledad Simoncini,2014). Little mention is made in the literature of albumen as a potential source of Ca or P. Ca must be moved directly from the yolk to the developing embryo, or must be mobilized from the shell across the chorioallantoic membrane (Packard & Clark,1996). The ratio of each egg component's contribution of Ca to the foetus varies with species (Packard & Packard,1989; Stewart & Ecay,2010). In *A. mississippiensis*, Packard and Packard (1989) showed that most Ca originates from the shell, but that yolk may serve as a temporary store of shell-derived Ca, so that the residual yolk at hatching contains as much as or even more Ca than that of the freshly laid egg. Jenkins (1975) described the mineral content of a small sample of unbanded and fertile *Crocodylus novoguinae* eggs and Packard and Packard (1989) studied the foetal uptake of Ca by fertile *A. mississippiensis* eggs. No similar studies have been performed on *C. niloticus* eggs. Fernández, Simoncini, and Dyke (2013) compared the microscopic appearance of normally and abnormally calcified *Caiman latirostris* eggshells, identifying two broad categories of abnormalities: fragile, insufficiently calcified eggshells, and well-calcified eggshells with abnormal shell surface calcification.

The researchers stated that eggs with insufficiently calcified shells had a poor hatching rate, whilst those with surface abnormalities did not differ from normal hatching rate; however, the concentration and content of Ca in the eggshell were not measured in this study.

If pathologies relating to the Ca and P content of eggs are to be investigated, or if research trials involving dietary or other modifications to husbandry regimens are to be properly planned, it is important that the correct samples are collected. For this reason, sample means and variances, as well as the grouping effect of clutch ('clutch effect'), must be described for the Ca and P concentration and content of each egg component. Furthermore, potential confounding effects must be quantified. In the course of normal incubation of a fertile egg, an opaque band forms around its lesser circumference due to development of the allantochorion beneath the shell membrane. The growth of this band can be used to determine fertility status and assess the progression of embryonic development in the first half of incubation. Eggs that fail to form such a band may never have been fertilized, or the embryo may have died at a very early stage of development. For the present research, it was assumed that such unbanded eggs were reflective of the egg composition of freshly laid fertile eggs prior to changes effected by a developing embryo. In summary, the research described here had the following aims:

1. To describe the concentration and content of Ca and P in each unbanded egg component, and measure the grouping effect of clutch on these variables.
2. To assess the effect of the following potential confounding variables on the content of Ca or P in each unbanded egg component: (1) clutch size, (2) date of laying, (3) female's pond of residence, (4) component wet mass and (5) component Ca or P concentration.

2 MATERIALS AND METHODS

2.1 Ethics statement

All procedures were approved by the University of Pretoria Animal Ethics Committee (certificates v104-16 and v105-16).

2.2 Specimen collection and preparation

Eggs were collected on a farm in the North West Province, South Africa. Female breeder crocodiles were fed a mixture of crocodile carcasses from an on-farm abattoir, and chicken carcasses enriched with a vitamin, omega-3 fatty acid and micromineral supplement. No information was available on the Ca and P content of breeder female diet. Females were communally housed in one of five breeder ponds, each surrounded by earthen banks for basking and egg-laying. As part of routine farm management, clutches were dug up shortly after they were laid and were artificially incubated buried beneath vermiculite in polystyrene incubation boxes. Clutches were not identified to the female that laid them.

Unbanded eggs (n = 967) were collected once viable eggs within the incubation box had hatched. Eggs were cleaned using a moist gauze swab, opened, and their components separated and weighed as detailed previously by Brown, Forbes, Myburgh, and Nöthling (2019). Of all eggs collected, only those 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 (Labcon, Ferndale, South Africa), after which the two were manually separated and their respective dried masses determined.

Direct measurement of the individual wet mass of unbanded egg shell and shell membrane could not be performed, because repeatable, clean separation of unbanded wet shell and shell membrane was not possible, a finding also noted by Ferguson (1982) for *A. mississippiensis* eggs. For another research study (manuscript in preparation), the shell and shell membrane of 30 fertile eggs were separated whilst wet (it was found that separation is much easier in fertile eggs). The wet shell and shell membranes of fertile eggs were weighed, dried, weighed again and their dry mass fractions measured. Since unbanded and fertile egg shells and shell membranes were macroscopically highly similar in appearance and texture, the mean dry mass fraction of these 30 fertile egg shells was used as a proxy for the dry mass fraction of unbanded shells. This figure, together with each unbanded egg shell's dry mass, was used to obtain an estimate of its wet mass. A similar process was followed for shell membranes.

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 fraction of yolk and albumen determined.

2.3 Sample analysis

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

2.3.1 Sample digestion and analysis

Approximately 0.2 g of each sample was weighed into separate Teflon digestion tubes. 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 whilst swirling at room temperature, and 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) 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 solution was determined by subtraction. One millilitre of this solution was aspirated with a Rainin variable micropipette (Mettler Toledo, Greifensee, Switzerland), and its weight determined in grams 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:

$$\text{Volume} = ((\text{Combined mass of container and sample}) - (\text{Container mass}))/(\text{density of liquid}).$$

An Agilent 5100 (Agilent Technologies) inductively coupled plasma, optical emission spectrometer (ICP-OES) with an autosampler was used for analysis. Sample concentration was determined from the mean of three replicates.

Ca and P standards (Inorganic Ventures) diluted to concentrations of 1, 10, 20, 50, 100, 250 and 400mg/L were used. A 1%HNO_3 solution was used as a sample blank. A calibration curve with an *R*-squared value of .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).

2.3.2 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). Certified concentrations for Ca were 110.70 g/kg with an allowable variation of 5.30 g/kg, 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, and samples of KH_2PO_4 equivalent to 227.59 g/kg P and $CaCO_3$ equivalent to 400.44 g/kg Ca were analysed after every 20 samples to check for machine 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) amongst submitted samples.

2.3.3 Specimen Ca and P determination

One hundred and eighty-five unbanded eggs had a clear albumen (clarity score 1). The concentrations of Ca and P were measured in the shell membrane, yolk and albumen of 95 of these eggs from 78 clutches. In one to three components amongst these 95 eggs, the concentration of Ca or P was, however, not measured. A pilot sample run showed very low variability in shell Ca concentration and negligible shell P concentration, so to save costs, only 29 shell samples (from 15 clutches) were analysed. Element concentration in these analysed samples, together with the original wet masses of 15 shells, 77 shell membranes, 42 yolks and 42 albumens was used to determine the content of the element in each component.

2.4 Data analysis

Stata 14 (Statacorp) was used for statistical analysis.

2.4.1 Assessment of measurement accuracy

The mean, *SD* and CV were determined for each set of reference material results and compared with certified values. The intra-assay CV was determined for each duplicate sample pair, and the mean of these CVs was calculated for each egg component.

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

Where data from a single egg per clutch were available, that egg was taken as representative of its clutch. Where data from more than one egg per clutch were available, a single egg was randomly selected per clutch. For Ca and P concentration and content, normality of distribution was assessed. 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 non-normal, the median, 25th and 75th percentiles were used. The proportional contribution of each component to total egg Ca and P was described. After selecting those clutches with data from two or more eggs, the intracluster correlation coefficient (r_{ic}) was determined for each component's Ca and P concentration and content.

2.4.3 The effect of covariates on content of Ca and P in each component

Using one randomly selected egg from each clutch where egg component Ca and P content was determined, the Ca and P content of each component other than egg shell was graphed using histograms and visually inspected for normality and the Stata command 'sktest' was used to assess normality of distribution at the 5% significance level. If the histogram and sktest suggested that the content of Ca or P in a component was non-normally distributed, a log (base 10) transformation was performed. If log transformation failed to normalize the distribution, extreme outlying data points (greater than 3 *SD* from the mean) of the transformed variable were excluded. First, a multilevel mixed-effect model was used, with clutch as a stochastic second level grouping variable and with the predictor variables clutch size, date of laying, female's pond of residence, component wet mass and component Ca concentration. Then, a likelihood ratio test was used to compare this model to a multiple regression model without considering the clutch effect.

If the likelihood ratio test was not significant ($p > .05$), a multiple regression model was used, without clutch as grouping variable. All regression models were checked for normality and heteroscedasticity of residuals (Breusch–Pagan/Cook–Weisberg test). In all regression models, non-significant covariates were sequentially removed from the model. Only significant coefficients, together with their *p* values, standard errors, 95% confidence interval and *t* statistic were reported.

3 RESULTS

3.1 Assessment of measurement accuracy

Mean CRM Ca measured 7.95 g/kg (7.2%) lower than the mean certified concentration (measured mean = 102.75 g/kg, *SD* = 1.26, *n* = 26), whilst mean CRM P measured 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), whilst 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).

As shown in Table 1, the concentration of Ca and P varied more between duplicate samples of shell membrane than it did amongst duplicate samples of albumen or yolk. Duplicate samples of shell had consistent Ca concentrations. No P was detectable in any shell samples.

TABLE 1. Summary of average intra-assay CV (%) of duplicate Ca and P concentration measurements

	Ca	P	
	CV	CV	*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 not detected in shell samples.

[b] P was not detected in three of 22 shell membrane samples submitted for determination of intra-assay CV.

3.2 Concentration and content of Ca and P in each component

Descriptive statistics for the concentrations of Ca and P in each component are shown in Table 2. Of all components, shell Ca concentration was by the far the highest and least variable. Shell membrane had high, but variable concentrations of Ca and P. Albumen had low, but relatively variable concentrations of both Ca and P. Yolk had the second highest concentration of Ca, with little variability, and the highest concentration of P, again with little variability.

TABLE 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	–	–	19.75	10.05	30.22	77
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.

The content of Ca and P (in grams) per component is shown in Table 3, and the relative % contribution is shown in Table 4. Shell contributed by far the greatest amount of Ca but contributed no P. Most Ca not contributed by the shell was contributed by the yolk. The yolk contributed almost all the P found in the unbanded egg. Shell membrane and albumen contributed very little Ca and P.

TABLE 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	–	–	0.031	0.014	0.045	77
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.

TABLE 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 r_{ic} for Ca and P concentration and content is summarized in Table 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. A strong clutch effect existed for shell Ca content and yolk Ca and P concentration and content. A very weak clutch effect existed for shell Ca concentration, and albumen Ca and P concentration.

TABLE 5. Intracluster correlation coefficients (r_{ic}) for the concentration and content of Ca and P in each egg component

	r_{ic}	*n* eggs	*n* clutches
Ca concentration			
Shell	.39	29	15
Shell membrane	.63	28	14
Albumen	.12	32	15
Yolk	.79	32	15
P concentration			
Shell membrane	.59	29	15
Albumen	.17	32	15
Yolk	.76	32	15
Ca content			
Shell	.87	29	15
Shell membrane	.75	28	14
Albumen	.20	14	8
Yolk	.64	16	9
P content			
Shell membrane	.69	29	15
Albumen	.15	16	9
Yolk	.68	16	9

3.3 Effect of covariates on Ca and P content

Clutch size and pond had no effect on the Ca or P content in any component ($p > .05$). Covariates influencing the Ca or P content of each component included the Ca or P concentration and the mass of the component, and in one case, date of laying.

3.3.1 Shell Ca content

A likelihood ratio test showed no advantage to using a multilevel model over a simple regression model for shell Ca content ($p = .055$). As illustrated in Figure 1a, the regression model showed that estimated wet shell mass had a significant association with shell Ca content, whereas shell Ca concentration did not: controlling for other covariates, for each gram increase in wet egg mass, shell Ca content increased by 0.27 g ($SE = 0.02$, 95% CI = 0.23–0.32, $df = 21$, $t = 12.25$, $p < .001$).

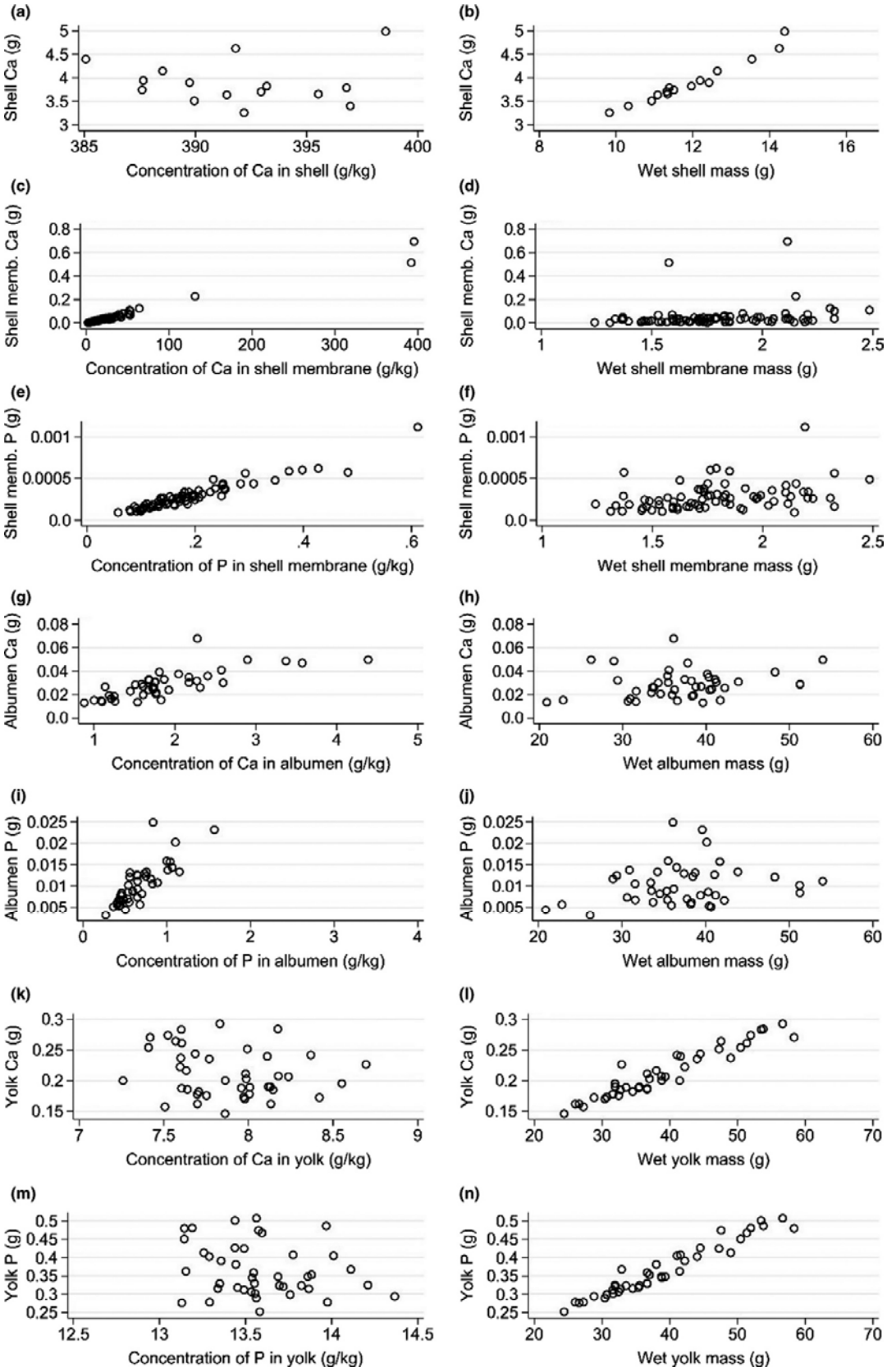

FIGURE 1. Association between the content of Ca or P and its concentration (left column) or its mass (right column) for each component of Nile crocodile eggs

3.3.2 Shell membrane Ca and P content

As shown in Figure 1c–f, shell membrane Ca and P content had a more obvious linear relationship with the element concentration; however, the component mass was also a statistically significant covariate.

The regression model showed that, controlling for other covariates, the log of shell membrane Ca content was estimated as −5.04 + 0.24(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 = 0.007, 95% CI = 0.23–0.25, t = 34.47, $p < .001$; *SE* of coefficient of log of shell membrane Ca concentration= 0.005, 95% CI = 0.99–1.01, t = 194.71, $p < .001$).

The regression model showed that, controlling for other covariates, the log of shell membrane P content was estimated as −3.98 + 0.24(wet shell membrane mass in grams) +1.02(log of shell membrane P concentration in g/kg). (*SE* of coefficient of wet shell mass = 0.007, 95% CI = 0.23–0.25, t = 36.66, $p < .001$; *SE* of coefficient of log of shell membrane P concentration = 0.008, 95% CI = 1.00–1.03, t = 129.76, $p < .001$).

3.3.3 Albumen Ca and P content

Controlling for other covariates, for each increase by 1 g/kg in albumen Ca concentration, albumen Ca content increased by 0.01 grams (*SE* = 0.001, 95% CI = 0.01–0.02, t = 11.82, $p < .001$; Figure 1g). Wet albumen mass (in grams) was also predictive of albumen Ca content: controlling for other covariates, for each gram increase in albumen mass, albumen Ca content increased by 0.006 g (*SE* = 0.001, 95% CI = 0.007–0.016, t = 5.44, $p < .001$; Figure 1h).

Albumen P concentration had a significant effect on albumen P content: controlling for other covariates, for each increase by 1 g/kg in albumen P concentration, albumen P content increased by 0.014 g (*SE* = 0.001, 95% CI = 0.012–0.017, t = 14.20, $p < .001$; Figure 1i). The wet mass of albumen (in grams) had a tiny but statistically significant effect on total albumen P: controlling for other covariates, for each gram increase in albumen mass, albumen P content increased by 0.0002 g (*SE* = 0.00004, 95% CI = 0.0001–0.0002, t = 4.53, $p < .001$; Figure 1j).

Date of laying had a statistically significant effect on albumen P content: controlling for other covariates, for each additional day within the laying season, albumen P content decreased by 0.00006 g (*SE* = 0.00003, 95% CI = −0.0001 to −0.00001, t = −2.51, $p < .05$). In practical terms, this meant that an egg from a clutch laid on the last day of the laying season could be expected to contain on average 0.003 g less P than one laid forty-six days earlier on the first day of the laying season.

3.3.4 Yolk Ca and P content

Controlling for other covariates, the Ca content of the egg yolk 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, t = 6.15, $p < .001$; Figure 1k) and 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, t = 18.6, $p < .001$; Figure 1l).

Controlling for other covariates, the P content of yolk increased by a mean of 0.03 g for each increase by one g/kg in yolk P concentration (SE = 0.009, 95% CI = 0.012–0.048, t = 3.43, $p < .05$; Figure 1m) and by 0.008 g for each gram increase in wet yolk mass (SE = 0.0005, 95% CI = 0.007–0.009, t = 18.32, $p < .001$; Figure 1n).

4 DISCUSSION

On commercial crocodile farms, unbanded eggs are freely available specimens with the potential for use in diagnosis and study of problems facing captive hatchling production, an observation made by Leiva, Labaque, Fernandez, Piña, and Simoncini (2018) who studied differences in physical characteristics and yolk fatty acid composition between unbanded and fertile *C. latirostris* eggs. Amongst archosaurs, embryonic Ca metabolism has been studied in greatest detail in the domestic fowl (Akins & Tuan, 1993; Terepka, Coleman, Armbrecht, & Gunther, 1976; Tuan, 1979). A very little crocodilian research is available on the topic; however, Packard and Packard (1989) found in *A. mississippiensis* and Jenkins (1975) found in *C. novaeguineae* that the shell contributes substantially to embryonic Ca via transport across the chorioallantoic membrane. Manolis and Webb (2016) reported clinical Ca deficiency in crocodiles fed red meat without supplementary Ca as well as animals housed under cover and unable to synthesize vitamin D_3. It is conceivable that maternal dietary Ca deficiency may translate to reduced deposition of Ca in the egg as suggested by Lance et al. (1983), but such an effect in crocodiles has not been formally or even anecdotally reported. Furthermore, the effect of subnormal egg Ca and P content on embryo development and viability is unknown. It has long been known that environmental toxins such as the organochlorines (Lundholm, 1997) can interfere with Ca deposition in the shell gland. Stoker et al. (2013) found reduced pore density in *Caiman latirostris* eggs exposed to organochlorine compounds and posited this as a potential cause of reduced hatchability. Such environmental intoxication was mentioned by Fernández et al. (2013) as a potential cause of abnormal eggshell calcification. Therefore, in addition to the obvious economic motive of more healthy hatchlings per female, in-depth research into Ca and P metabolism in crocodile eggs could assist in understanding and gauging environmental intoxication by these compounds.

Unsurprisingly, the present study confirms prior findings for other crocodilian species: namely that most Ca is deposited in the shell and secondly in the yolk, almost all P is contained within the yolk (Packard & Packard, 1989), and at hatching a large quantity of Ca is discarded with residual shell (Stewart & Ecay, 2010). Therefore, shell and yolk should be collected when investigating embryonic Ca and P metabolism and pathologies thereof. In the present study, albumen contained on average less than one per cent of total egg Ca and less than three per cent of total egg P, suggesting that it is an insignificant source of these elements for the developing embryo. Shell membrane was found to have a highly variable Ca and P concentration, suggesting possible contamination by yolk or shell. In the unbanded egg, the shell membrane is a metabolically inactive layer, so a low Ca and P concentration may be expected. The situation in the actively metabolizing fertile egg may prove otherwise but remains to be assessed.

Low variability in shell Ca concentration across clutches, as well as a low r_{ic}, suggests that a small number of randomly sampled eggs, without regard to their clutch of origin, is a fair sampling strategy to determine the mean Ca concentration of eggshells in a population. Furthermore, since the shell of unbanded eggs was found to have a similar Ca concentration to pure $CaCO_3$, if analytical equipment is unavailable, the Ca content of shell may be estimated simply by multiplying the concentration of Ca in dry $CaCO_3$ (approximately

400 g/kg) by the dry mass of the shell. The high r_{ic} seen for shell Ca content was likely due to the strong grouping effect of clutch on shell mass (Brown et al., 2019), which, in turn, positively correlated with shell Ca content (Figure 1b).

The grouping effect of clutch on yolk Ca and P content was strong (r_{ic} of .64 and .68 respectively). As for shell Ca content, this may be because of the strong grouping effect of clutch on wet yolk mass (Brown et al., 2019), which, in turn, was strongly correlated with yolk Ca content (Figure 1l and n). This means in practical terms that a small number of yolk specimens per clutch, from as many clutches as possible, should be collected for future studies into yolk Ca and P content.

The most important factors influencing the Ca and P content of unbanded egg components were element concentration and component mass. 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. Eggs laid later in the season contained very slightly less albumen P than those laid earlier in the season; however, the very small difference of 3 mg from beginning to end of season casts doubt on actual biological significance. Eggs used for regression analyses came from five different breeding ponds. Although pond was included as a factor in all regression models and was found in all cases to be a non-significant covariate, two ponds contributed only seven eggs each to the total number of specimens, one pond contributed 15 eggs, a fourth contributed 20 and the fifth contributed 46. Ideally, samples per pond should be larger and more evenly distributed amongst ponds. Furthermore, unmeasured variables that differ from female to female are confounded with those seated in ponds, since each female lived in one pond only. A shortcoming of the present study is that it was not possible to trace a clutch of eggs to the female that laid it. This would have allowed for the evaluation of other covariates such as size and age.

Reference material Ca and P concentrations measured consistently lower than certified values. This systematic error may have been caused by issues in sample processing and analysis, including incomplete sample digestion, incomplete sample nebulization 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 whilst absolute values may be under-reported, the relative values are useful in comparing Ca and P in different egg components.

Findings from the present study will assist in planning of future studies. For example, the pattern of movement of Ca and P from the components of the freshly laid Nile crocodile egg to the embryo has yet to be determined. The effect of maternal diet on egg Ca and P content, and subsequent embryo viability must be evaluated, and a comparison between captive and healthy wild populations may be instructive in identifying shortcomings in captive crocodile husbandry.

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Conflicts of interest

None.

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