

**Nile crocodile (*Crocodylus niloticus*): Egg mass relative to egg component mass in unbanded and fertile eggs**

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## 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.

### **Keywords**

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

## 1. 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 (Thorbjarnarson, 1999, Dzoma et al., 2008). In South Africa, captive-laid eggs are the only legal source of farmed hatchlings (IUCN, 1996).

Captive production of Nile crocodile hatchlings is relatively successful (Khosa et al., 2012) but poorly studied. Early studies of unbanded and fertile *C. niloticus* eggs were broadly descriptive (Pooley, 1969) and confined to wild populations (Bigalke, 1931, Modha, 1967).

Production of healthy hatchlings dictates the success of farming with crocodylians. Nelson et al. (2010) showed that yolk mass, as well as albumen mass, are positively related to the mass of American alligator eggs, although albumen mass increases more rapidly with an increase in egg mass. They also showed that the mass of American alligator fetuses 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 calcium 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 mass could be ascribed to differences in their yolk-free body mass and the mass of

their intra-abdominal yolk. 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 intraclass 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 *Crocodylus porosus*, the variation of egg size among clutches is larger than that within clutches (Garnett and Murray, 1986). The same is true for *Alligator mississippiensis* (Deitz and Hines, 1980). These studies, as well as those of Stoker et al. (2013) on *Caiman latirostris* and Pooley (1962) on *Crocodylus 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.

## 2. Materials and methods

The University of Pretoria Animal Ethics Committee approved the study (certificates v104-16 and v105-16).

### 2.1. 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 (31 from 31 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 hours prior to processing, which occurred within 48 hours 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 that we used and reported as “egg mass”.

## 2.2. 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 sealed in ziplock 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 1). 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.

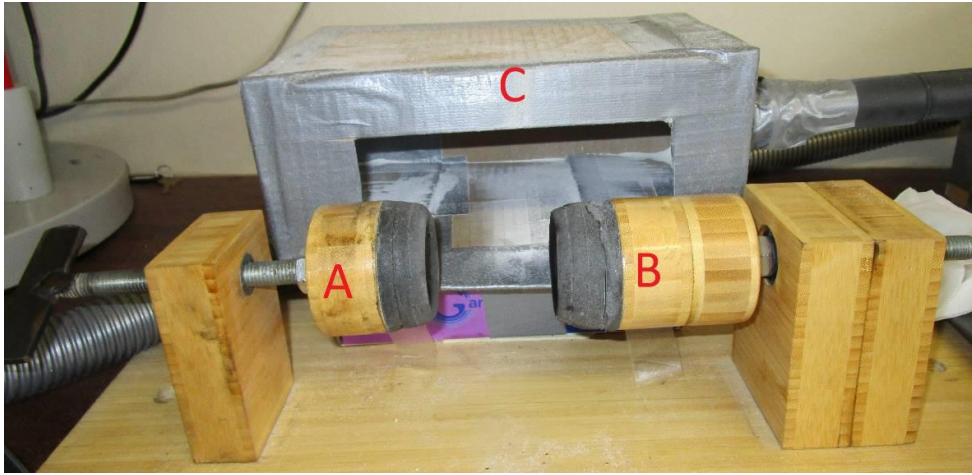


Figure 1: Device for opening of unbandaged eggs. Note padded head- and tailstocks (A and B), and dust collection shroud C.

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 (Figure 2).



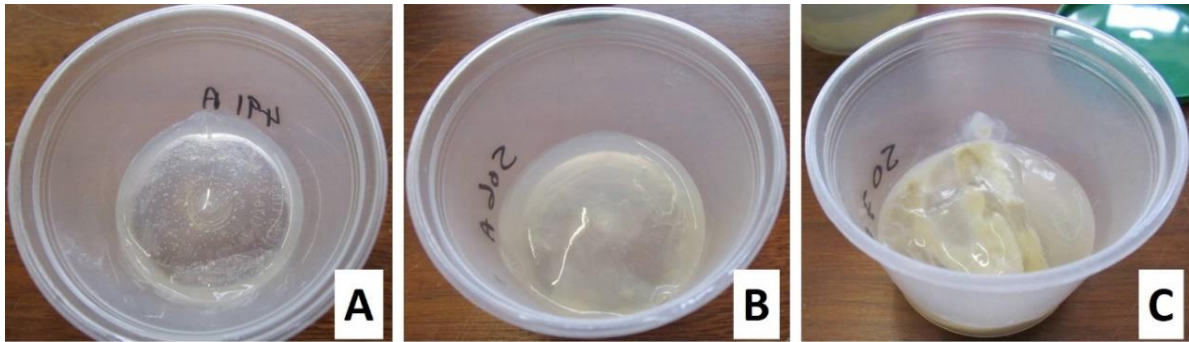


Figure 2: Albumen clarity scores of 1 (A), 2 (B) and 3 (C)

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

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 ziplock 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 ( $n = 31$ ) was divided by its wet mass, both of which were explicitly measured (see 2.3 below). 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.

### 2.3. Fertile eggs

For each fertile egg (n = 31), 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.

By the time each foetus was removed from its egg, it had been kept at or below 4 °C for at least 24 hours. 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 mass of eggshell and shell membrane were measured. Once separated, all fertile egg components were freeze- and oven dried, and their dry masses measured.

## 2.4. 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.

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

#### 2.4.1.1. 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 reported.

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

#### 2.4.1.2. 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.

Simple 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.

#### 2.4.2. Comparing component masses of unbanded and fertile eggs (aim 2 of the study)

For each of 29 fertile eggs, 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.

#### 2.4.3. 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.

2.4.4. 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).

We assessed variables that could confound the masses of eggs and their components, 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. Results

#### 3.1. Masses of egg components relative to egg mass

##### 3.1.1. 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 1 and Table 2. All variables were positively skewed, with the exception of wet albumen mass, which was negatively skewed.

Table 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 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), with the equation:

$$\text{Volume in millilitres} = 0.26 + 0.88(\text{egg mass in grams})$$

(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.

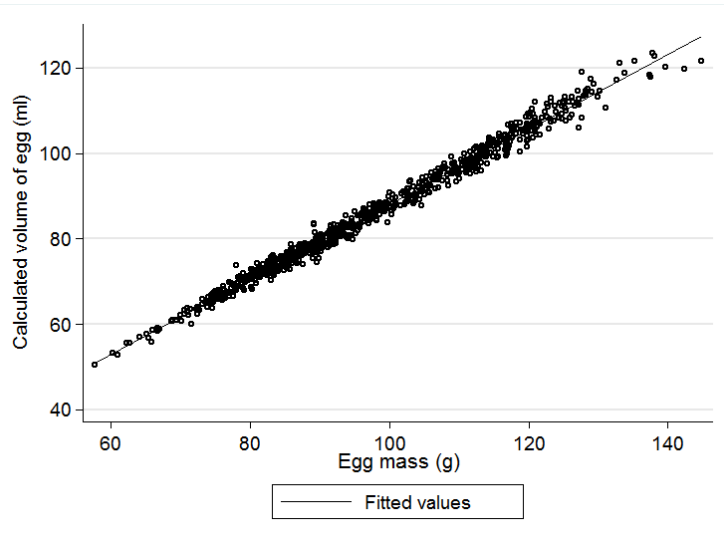


Figure 3: Relationship between egg mass and egg volume, calculated as the volume of an ellipsoid

### 3.1.2. Masses of fertile eggs and their components

Of 31 fertile eggs collected, one egg contained a runted foetus that had failed to internalise its egg yolk, and was excluded from all analyses. A summary of the masses of fertile egg components and their mass ratios are shown in Table 3 and Table 4.



Table 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 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 <sup>a</sup>	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

<sup>a</sup> 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 the others 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 4a). 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 =  $-39.4 \text{ g} + 0.50(\text{incubation period}) + 0.70(\text{egg mass})$ . 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 4b 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 =  $-9.75 + 0.26(\text{fertile egg mass})$ . 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 4c). Incubation period, which had no significant effect ( $P = 0.67$ ) was removed from the model. The regression equation was:

Isolated foetal mass =  $2.15 + 0.46(\text{egg mass})$ . 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 ( $P = 0.40$ ).

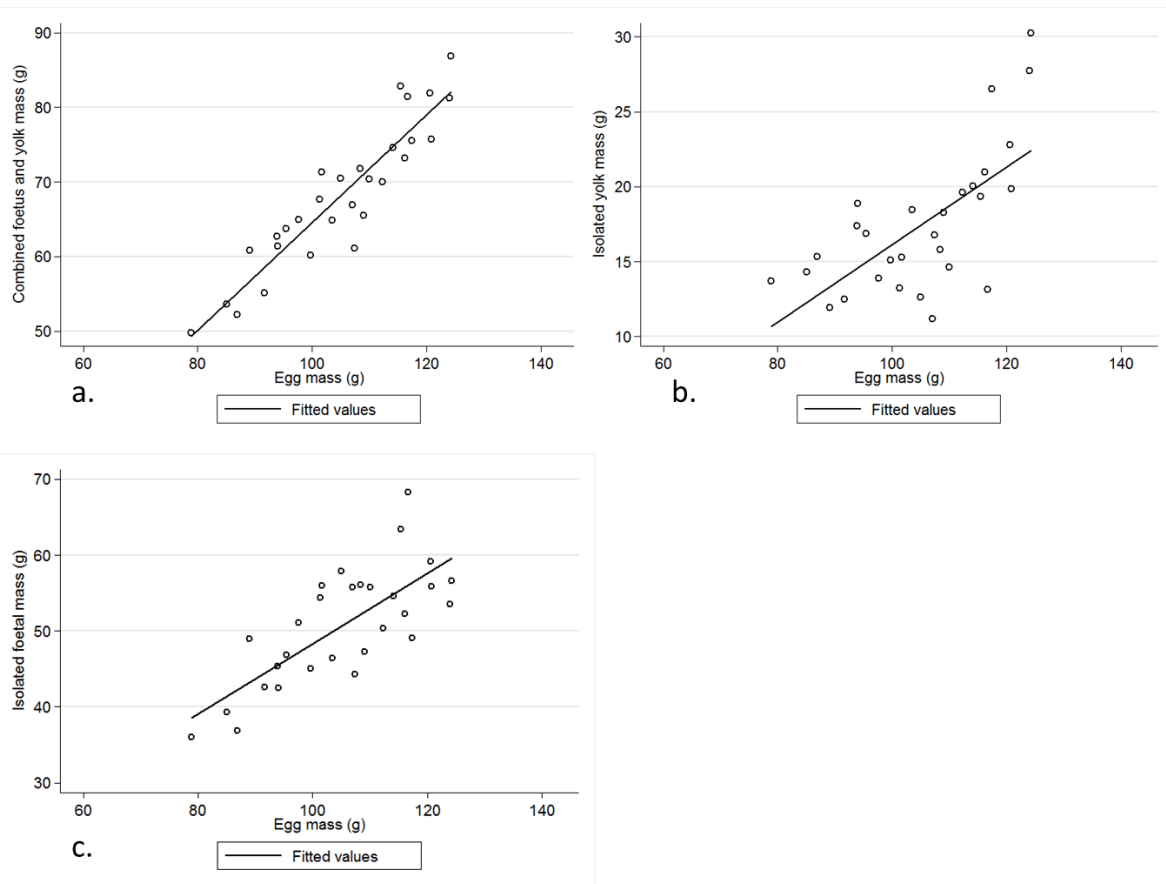


Figure 4: Association between total egg mass and (a) combined mass of foetus and intra-abdominal yolk, (b) intra-abdominal yolk, and (c) and yolk-free foetus.

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:

$$\text{Isolated yolk-free foetal mass} = -42.47 \text{ g} + 0.54(\text{incubation length}) + 0.73(\text{egg mass}) - 1.10(\text{yolk mass}).$$

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),  $P < 0.001$ ).

### 3.2. 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 -0.06 to 1.04) lower ( $t = 1.82$ , df = 28,  $P = 0.04$ ) (Figure 5).

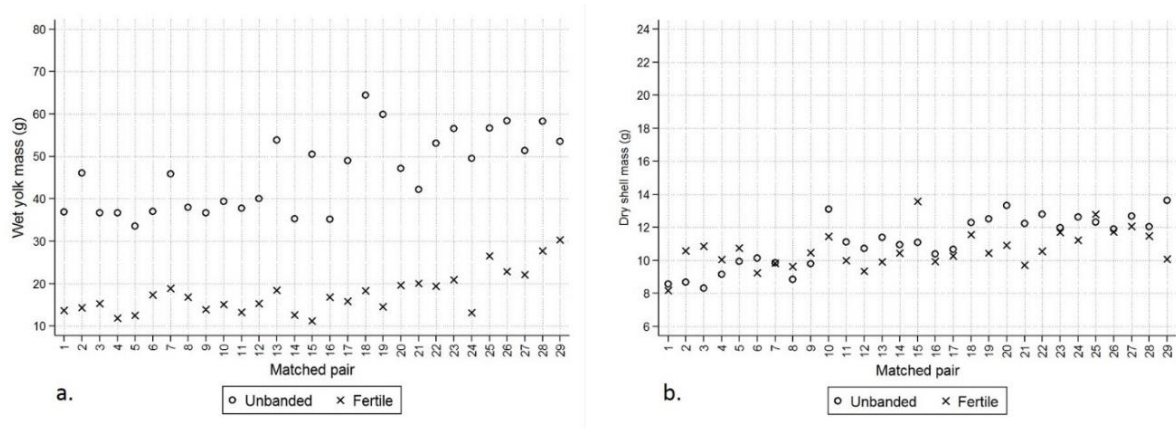


Figure 5: Comparison of masses of wet yolk (a) and dry shell (b) for fertile and unbanded size-matched eggs

### 3.3. The clustering effect of clutch on the masses of unbanded eggs and their components

#### 3.3.1. 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 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 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 6 and Figure 7 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 5 and the relatively small within-clutch ranges shown in Table 6.

Table 5: Intraclass correlation coefficients ( $r_{ic}$ ) 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 6: Summary of within-clutch ranges in the masses of unbanded eggs and their components

Component	Range in mass (g)				Clutches
	Min	Median	Maximum	IQR	
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

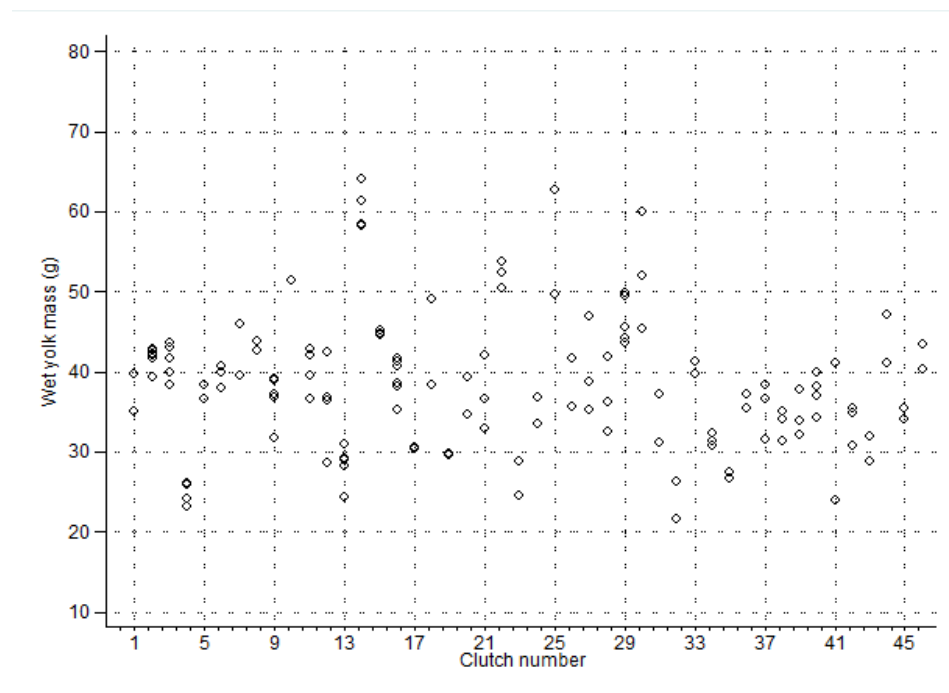


Figure 6: Wet yolk mass of each egg in each of 46 clutches containing at least two eggs with a clarity score of one



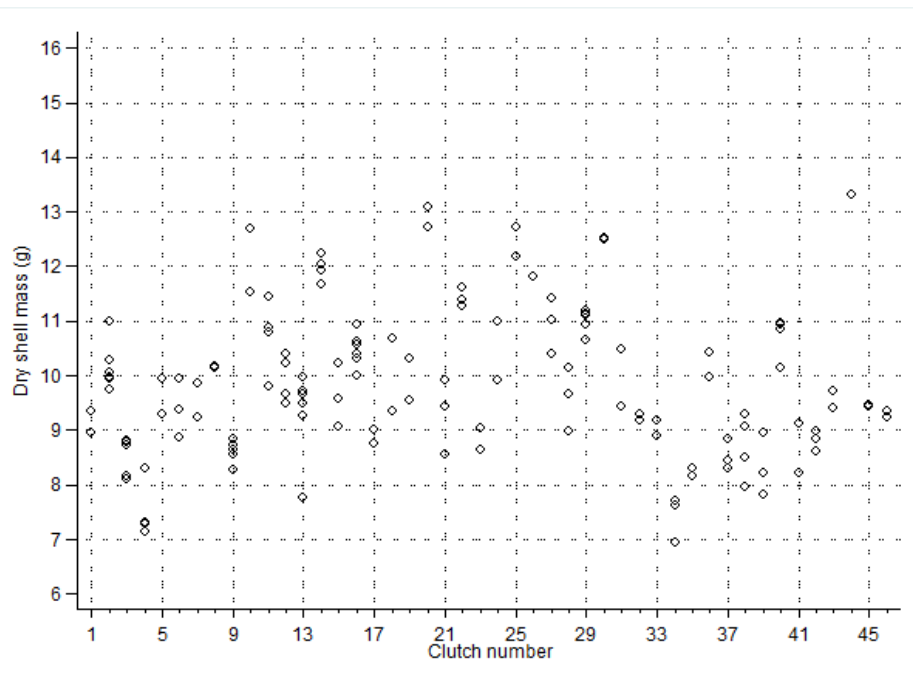


Figure 7: Dry shell mass of each egg in each of 46 clutches containing at least two eggs with a clarity score of one

### 3.4. Relationship between the mass of unbanded eggs and their components, and the effect of potential confounding variables

#### 3.4.1. 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,  $z = -2.51$ ,  $P = 0.01$ ).

#### 3.4.2. 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:

$$\text{Wet yolk mass} = -6.68 + 0.50(\text{egg mass}).$$

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,  $z = 14.98$ ,  $P < 0.001$ ).

#### 3.4.3. 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 mass of eggs laid by females in pond 2 is 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, the dry shell membrane mass is 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 mass 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,  $z = 3.08$ ,  $P = 0.002$ ).

#### 4. 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*. Our 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 (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 showed in the American alligator by Ferguson (1982).

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

This 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 *Crocodylus 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 *Caiman 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*.

Our findings suggest that the period within laying season does not have to be considered when collecting *Crocodylus 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 a higher male: female ratio may result 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).

We cannot explain 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.

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## **7. Declaration of conflicts of interest**

None

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