CHAPTER 46

Crocodile Egg Chemistry

S. Charlie Manolis[1,2], Grahame J. W. Webb[1,2] and Karen E. Dempsey[2]

THE amniote egg of birds (Romanoff 1960), chelonians (Ewert 1979, 1985) and crocodilians (Ferguson 1985) contains a large yolk mass which does not cleave [telolecithal eggs (Romer 1970)], and, relative to egg size, a very small embryo or embryonic primordium at the time of egg-laying. The egg contents supply the developing embryo with most of its nutrients, while the external nest environment sets the temperature of incubation and provides a medium for gas and water exchange. Embryos of these three groups develop the same extra-embryonic membranes — amnion, chorion and allantois — which partition the egg into similar compartments.

Knowledge of the basic chemistry of development in the eggs of birds has been available for many years (see Needham 1963; Romanoff 1960, 1967; Romanoff and Romanoff 1949), but comparable data for reptiles are virtually non-existent [notwithstanding the recent upsurge of interest in reptilian embryology in general (Gans and Billett 1985; Gans *et al.* 1985)]. Yet the factors which bring about the changes within reptile eggs are becoming increasingly important. Temperature-dependent sex determination is now known to be a common trait in crocodilians and turtles (see Ferguson Chapter 49; Webb *et al.* Chapter 50). Post-hatching growth and survivorship of crocodilians is intimately and perhaps independently associated with the temperature of egg incubation (Hutton 1984; Joanen *et al.* Chapter 51; Lang Chapter 30; Webb *et al.* Chapter 50). Nutritional deficiencies in the diet of adult female *Alligator mississippiensis* appear to lead to deficiencies in eggs and embryos (Joanen and McNease Chapter 32; Lance Chapter 42). Water exchange between crocodilian eggs and their environment may be related to post-hatching fitness (Grigg Chapter 48).

We are unable to say whether all or any of these factors are influenced by the chemical environment in which the embryos exist within the egg, because there is simply a paucity of information on what that environment is, how it changes during incubation, how it is affected by incubation temperature, and, how it is affected by water losses or gains.

This chapter describes the changing composition of egg contents that occurs during embryonic development in the Australian freshwater crocodile *Crocodylus johnstoni*. Particular attention is paid to: the egg contents prior to embryonic development; the utilization of egg contents with time; the concentrations of major ions in egg fluids; the concentrations of major excretory products in egg fluids; the effects of varying incubation temperature; and, the effects of varying levels of water loss. Where possible, the results obtained for crocodilians are compared with the comprehensive information available for birds and the scattered information available for other reptiles. Nitrogenous excretion and the possible role of ions in facilitating water movement within compartments of the crocodilian egg are discussed separately.

The results were obtained from the same eggs used for quantifying the effects of incubation temperature on embryonic development rate and sex determination (Webb *et al.* Chapter 50), and because of this, detailed analyses of the composition of embryos was not possible (they were fixed for histological examination). Similarly, for logistic reasons analyses were restricted to those that could be carried out on frozen samples. Accordingly, although the results greatly enhance our understanding of the structure and dynamics of embryological development in crocodilian eggs, and perhaps reptiles in general, they are by no means complete.

METHODS

General

Eggs were located and collected on a daily basis during the egg-laying season (in 1983) as described previously (Webb *et al.* 1983a). They were returned to the laboratory where one egg from each clutch was opened and the embryo was aged on the basis of the number of somite pairs (Webb *et al.* Chapter 50). All eggs were weighed, measured, and divided among water-jacketed incubators set at: 28°C, 29°C, 30°C, 31°C, 32°C, 33°C and 34°C. Within the incubators, eggs were positioned in trays, within plastic bags [Method A incubation of Webb *et al.*

[1]School of Zoology, University of New South Wales, P.O. Box 1, Kensington, New South Wales 2033.
[2]Conservation Commision of the Northern Territory, P.O. Box 38496, Winnellie, Northern Territory 5789.
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Chapter 50], and temperatures were recorded from probes amongst the eggs. All probes were calibrated against a certified thermometer. Temperature rises due to metabolic heat were compensated for on a daily basis and stated mean temperatures are within ± 0.2°C.

At varying times throughout the incubation period eggs were removed from the incubators and the contents were separated. A small piece of shell from the top, but to one side of the midpoint (along the outer edge of the opaque band where applicable), was removed, and a pasteur pippette was used to extract fluid from the central part of the egg. Once some of this fluid was removed, and the position of the embryo could be seen, a larger piece of shell was cut from the top and the remaining fluids and other egg contents were removed. All contents were weighed (± 0.01 g), and where sufficient quantities were available, densities were determined by weighing known volumes in a plastic syringe. All egg contents were then frozen. Embryos up to about 32 g in weight (yolk-free; less than 80 days MA_{30} — see later) were weighed on an analytical balance (± 0.0001 g).

At irregular intervals throughout incubation, eggs were briefly removed from the incubators, weighed, and had the state of the opaque band recorded. Eggs that were translucent at the time of collection (collected on the day after the night of laying), had the state of the opaque band recorded daily until it became 'even' (see Webb *et al.* Chapter 43).

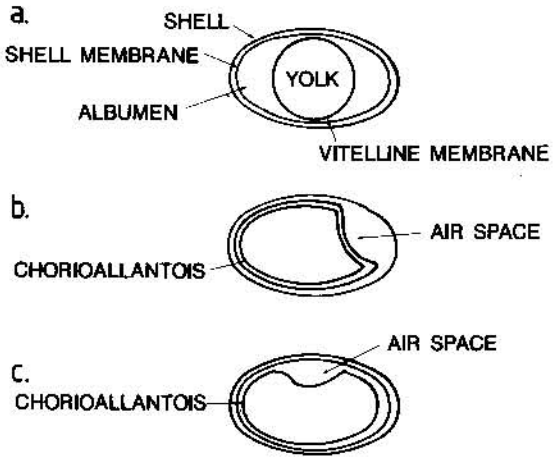


Fig. 1. Diagramatic cross-sections of a fertile crocodilian egg showing the main components *(a)*; an air space between the eggshell membrane and the eggshell *(b)*; and, an air space between the chorioallantois and the eggshell membrane *(c)*.

The presence of air spaces, either between the eggshell and eggshell membrane (Fig. 1b) or between the eggshell membrane and the chorioallantois (Fig. 1c) was noted. Although all eggs lost weight to some degree during incubation, those with air spaces, as a group, were considered to have suffered higher water losses than those without air spaces.

In many eggs, distinct amniotic and chorionic [sero-amniotic cavity of Patten (1925)] chambers were obvious, but this was not always the case. In some only one or the other was discernible, possibly reflecting a ruptured amnion or a lack of fluid within the chorionic chamber. As we could not discern the origins of these fluids confidently in all cases, most were analysed together as "chorio-amniotic" fluid.

Subembryonic, allantoic and chorio-amniotic fluids were analysed for concentrations of sodium, chloride, potassium, calcium, inorganic phosphate, bicarbonate, urea, soluble urate and creatinine using an 'Autolab' Ion Analyser. Osmolality of fluids was determined using a 'Knauer' osmometer. Dry weights of yolk, albumen, eggshell and shell membrane samples were recorded after 48 h drying at 60°C in a drying oven.

Lipid content of yolk was determined using the method of Bligh and Dyer (1959), with slight modifications as outlined by Fogerty *et al.* (1971). Following removal of the lipid fraction, the remaining protein pellet was dissolved in 2M NaCl and its protein content determined by the Biuret method (Young 1963). Preliminary analyses on albumen revealed negligible amounts of lipid, so only protein determinations were carried out. The minute amounts of lipid that may have been contained within the albumen did not appear to affect the colormetric method used.

In order to examine how the amounts of yolk, albumen, lipids, ions, proteins etc. changed with time, variation attributed to egg size was reduced by standardizing components to the mean *C. johnstoni* egg size of 68.2 g (Webb *et al.* 1983a). The major egg contents (yolk, albumen and eggshell) are in the same relative proportions independent of egg weight. Unless otherwise stated, errors mentioned with a mean are ± one standard error.

Ages, Stages and Morphological Ages

When analysing continuous embryological events which occur over time, analyses are reasonably straight-forward if the same incubation conditions are used; the time base can be straight "days of incubation" (*real age*; RA) or "percentage of total incubation time" (*percentage age*; PA), and variables of interest can be plotted against it. Embryological *stages* are of limited use for this purpose, as they are discrete measures which last for different amounts of time.

When development rate is varied greatly, by varying incubation temperature, a number of analytical problems can arise when attempting to identify temperature effects over and above those associated with development rate. *Percentage age* is one option for scaling or standardizing events independent of development rate, but the use of PA

with crocodilians is confounded to some degree by the embryos internalizing their yolk and hatching at different stages (early with fast development; late with slow development); 50% PA at 28°C refers to an embryo at a later stage of development than 50% PA at 34°C.

The approach we have taken for standardizing development rate is to quantify the relationship between embryo size and incubation temperature at 30°C, so that any embryo can be assigned an equivalent 30°C age on the basis of its size, and regardless of its real age. The larger hatchlings from low incubation temperatures can be assigned a 30°C age in days that is in excess of the age of hatching at 30°C. As this relative age scale is based on the morphology of an embryo, we term it *morphological age*, with a subscript "30" to indicate it is based on a 30°C incubation temperature (MA_{30}). (This subscript could of course be extended to include other aspects of the incubation environment). The derivation of coefficients used to predict back and forth between *real ages* and *morphological ages* for both *C. johnstoni* and *C. porosus* are in Webb *et al.* (Chapter 50).

RESULTS

The Basic Egg Components

General

Unlike bird ova, which pass individually through the oviducts and are shelled and laid rapidly, crocodilians and other oviparous reptiles store the full complement of ova in the oviducts. Here, over a period lasting several weeks or longer they are "packaged" into shelled eggs, and are eventually laid during a single, laying session. The oviducts probably contain sperm before ovulation, such that ova are fertilized as they reach and pass through the oviducts.

Embryological development and changes in the egg contents induced by the activity of the embryonic tissues begin while the ova are within the oviducts. By the time the egg is laid, significant changes have already occurred. Indeed, they may have occurred before the ova (yolk) becomes surrounded by albumen and is shelled. Infertile shelled eggs offer insights into the basic composition of an egg, uncomplicated by changes induced by embryonic tissues.

Approximately 4% of wild *C. johnstoni* eggs (Webb *et al.* 1983a) and 6% of wild *C. porosus* eggs (Webb *et al.* 1983b) are usually classified as infertile. This classification is based on a failure to develop an opaque band, lasting resistance to decomposition, and the division of the egg contents into yolk and albumen alone; there is no sign of subembryonic fluid (see below).

In reality, eggs classified as infertile on these criteria include real infertile eggs (for example sometimes almost exactly half a clutch is infertile, suggesting sperm did not penetrate one oviduct), and eggs in which the embryo died at a very early stage of development within the oviducts. For the purposes of defining the basic structure of a crocodilian egg before significant embryonic development has occurred, the distinction is probably unimportant.

Table 1. The relative proportions of yolk, albumen and shell (plus shell membrane) for the eggs of four species of crocodilians. The values for *Crocodylus johnstoni, C. porosus* (this study) and *C. novaeguineae* (Jenkins 1975) are from infertile eggs. Values are mean wet weights expressed as percentages of total egg weights. Errors are one standard error and sample sizes are in brackets.

	Eggshell	Yolk	Albumen
A. mississippiensis[1]	12.0 ± 0.3 (16)	40.6 ± 1.0 (17)	46.5 (16)
A. mississippiensis[2]	–	–	44
C. johnstoni	11.9 ± 0.3 (40)	46.0 ± 0.8 (37)	42.1 ± 0.8 (38)
C. novaeguineae[3]	14.0 ± 0.9 (9)	–	–
C. porosus	12.0 ± 0.4 (21)	42.4 ± 0.8 (21)	45.7 ± 0.8 (21)

[1]data from M. W. J. Ferguson
[2]Tracy and Snell 1985
[3]calculated from Jenkins 1975

Infertile crocodile eggs are composed of three main components: yolk, albumen and the shell. A thin vitelline membrane surrounds the yolk mass and a shell membrane surrounds the egg contents completely (Fig. 1a). In a fertile egg at the time of laying, the embryo is attached to the inner surface of the vitelline membrane (see Webb *et al.* 1986b, Chapter 43).

Relative proportions of yolk, albumen and shell (including the shell membrane) in infertile eggs of *C. johnstoni* and *C. porosus* are in Table 1. Formulae for predicting weights of egg contents from egg dimensions are in Table 2.

Yolk

Crocodylus johnstoni eggs contain proportionally more yolk and less albumen than do *C. porosus* and *Alligator mississippiensis* eggs (Table 1). The proportional yolk size (40.6-46.0%) is within the range of values derived from 17 species of turtles (31.9-55.0%; Ewert 1979; Thompson 1983; Tomita 1929; Simkiss 1962; Webb *et al.* 1986a), and is higher than in the eggs of precocial and altricial birds [30-40% and 15-20% yolk respectively (Romanoff and Romanoff 1949)]. Precocial birds are feathered and able to fend for themselves at hatching, whereas altricial birds are typically featherless and require considerable post-hatching attention from the parents.

Table 2. Formulae for predicting weight of egg contents (in grams) from egg dimensions for *Crocodylus johnstoni* (CJ) and *C. porosus* (CP) using linear regression analysis. Egg breadth (EB) and egg length (EL) are in centimetres. 'a' refers to the intercept and 'b' to the slope. SEE is the standard error of the estimate (Zar 1974). 'Shell' refers to eggshell with eggshell membrane. Low r^2s reflect the extent of variation relative to the relatively small size range.

To Predict	From	Species	a	b	SEE	r^2	N
Albumen	EB	CP	-67.01	23.65	3.20	0.67	21
Albumen	EB	CJ	-28.97	13.77	2.12	0.66	40
Albumen	EL	CP	-55.87	13.01	3.38	0.62	19
Albumen	EL	CJ	-31.68	8.73	2.64	0.50	38
Yolk	EB	CP	-49.51	19.28	2.25	0.74	19
Yolk	EB	CJ	-49.11	19.38	1.71	0.86	37
Yolk	EL	CP	-30.33	9.24	3.45	0.61	22
Yolk	EL	CJ	-33.45	9.38	3.58	0.39	38
Shell	EB	CP	-22.71	7.27	0.91	0.73	30
Shell	EB	CJ	-4.41	2.97	0.68	0.46	44
Shell	EL	CP	-11.17	2.98	1.14	0.58	30
Shell	EL	CJ	-4.41	1.81	0.74	0.37	43

Although such data are useful for examining gross differences between species and between groups, they can be highly misleading. Eggshell weights can represent markedly different proportions of egg weight, and in reality, the albumen and eggshell can perhaps better be considered as "essential packaging" for an ovum; biologically, ovum size may be a truly independent variable.

If viewed only as a proportion of the combined albumen and yolk weight, crocodilian yolk represents 52.2% (*C. johnstoni*), 48.1% (*C. porosus*) and 46.6% (*A. mississippiensis*) of egg contents, which is within the range for turtles (34-58%; Tomita 1929; Simkiss 1962; Ewert 1979; Thompson 1983; Webb *et al.* 1986a) and precocial birds (24 to 57%; Carey *et al.* 1980), but is proportionally larger than for altricial birds (14-32%; Carey *et al.* 1980). As in precocial birds (Romanoff and Romanoff 1949), the production of crocodilian young is associated with a relatively small amount of albumen being supplied to a yolk-laden egg. The *C. johnstoni* ovum is supplied with relatively less albumen than that of *C. porosus* and *A. mississippiensis*.

The mean density of *C. johnstoni* yolk (1.058 ± 0.003 g cm^{-3}) is not significantly different from that of *C. porosus* (1.042 ± 0.006 g cm^{-3}) nor that of *A. mississippiensis* (1.038 ± 0.009 g cm^{-3}). Crocodilian yolk has a similar density to turtle yolk (1.061 ± 0.002 g cm^{-3} for *Carettochelys insculpta*; Webb *et al.* 1986a) but is of greater density than the yolk of the domestic fowl (1.035 g cm^{3}; Romanoff and Romanoff 1949).

Water content of crocodilian yolk (Table 3) is similar to that of turtle yolk (51-68%; Ewert 1979; Thompson 1983), and is closer to the water content of altricial bird yolk (57-66%; Ricklefs 1977) than it is to that of domestic fowls and precocial birds in general (43-50%; Ricklefs 1977). The yolk of oviparous lizards (*Amphibolurus barbatus*) and snakes (*Coluber constrictor*), has an appreciably higher water content [78.4% and 70% respectively; Badham (1971) and M. J. Packard *et al.* (1984a) respectively]. As these eggs seem to lack albumen, the water content is perhaps more readily comparable with the total water content of crocodilian eggs.

Lipid and protein make up the bulk of the remaining yolk (Table 3), and on a wet weight basis, *C. johnstoni* yolk has relatively less lipid and slightly more protein than in the domestic fowl.

Some 47.0% of the dried mass of yolk in *C. johnstoni* eggs is in the form of lipids, whereas in six turtle species, dry weight lipid values ranged from 16-34% (Lynn and von Brand 1945; Chaikoff and Entenman 1946; von Brand and Lynn 1947; Needham 1963; Ricklefs and Burger 1977; Ewert 1979; Congdon *et al.* 1983; Thompson 1983). In the oviparous lizard *Morethia boulenger*, 27% of dry egg weight was lipid (Smyth 1974) which, on the basis of yolk weight, is within the range for turtles. Birds on the other hand typically have higher levels of yolk lipid than are found in crocodilians (55-65%; Ricklefs 1977).

Albumen

Albumen completely surrounds the yolk mass in infertile and freshly laid crocodile eggs (Fig. 1a), with a thin layer (3-4 mm deep) separating the yolk mass from the shell membrane around the middle of the egg. Most albumen is within the poles of the egg, a situation common to birds (Romanoff and Romanoff 1949; Romanoff 1967;) and turtles with elliptical eggs (Ewert 1979; Morris *et al.* 1983; G. C. Packard *et al.* 1983; G. C. Packard and M. J. Packard 1984). In the spherical eggs of some turtles, there is an even thickness of albumen surrounding the yolk (Webb *et al.* 1986a,b).

Table 3. The composition of crocodilian egg contents at the time of laying, compared with those of the domestic fowl (after Romanoff and Romanoff 1949).

	Albumen			Yolk		
	%water	%protein	%lipid	%water	%protein	%lipid
C. johnstoni	95.0	4.3	neg.	58.1	19.2	19.7
C. porosus	96.4	–	–	56.4	–	–
Gallus domesticus	87.8	10.5	0.02	48.7	32.6	16.8

The albumen of crocodilian eggs differs in some striking ways from that of avian eggs. There are no chalazae extending through to the yolk in crocodilian eggs (Ferguson 1982). The supporting role they play in bird eggs (Romanoff and Romanoff 1949) may be obviated by the thicker consistency of crocodilian albumen (see below), although crocodile eggs are not rotated during incubation and the embryos need to attach to the shell membrane (see Webb *et al.* Chapter 43); perhaps support is not necessary. Also, there appears to be no clear distinction between "thick" and "thin" albumen in crocodilian eggs. The albumen has a laminated structure in which the layers closest to the vitelline membrane contract with dehydration (see Webb *et al.* 1986b, Chapter 43). The occasional reference to thick and thin albumen in the literature seems to refer to albumen (thick) and subembryonic fluid (thin).

Albumen accounts for 42-47% of total egg weight in three species of crocodilians (Table 1), which is within the range for nine species of turtles with brittle-shelled eggs (34.4-48.0%; Ewert 1979; Thompson 1983; Webb *et al.* 1986a) and eight species with parchment-hard shelled eggs (39.2-61.9%; Tomita 1929; Simkiss 1962; Ewert 1979). Albumen appears to make up a larger proportion of egg weight in species with parchment-shelled eggs, because shell weights are reduced.

On the basis of egg contents alone (yolk and albumen), the amount of albumen in crocodilian eggs (47.8-51.9%) is similar to that in both brittle- (41.6-65.9%) and parchment-shelled (45.7-56.5%) turtle eggs. It is also within the range for precocial bird eggs (43-76%), but is well outside the range for altricial bird eggs (68-86%) (Carey *et al.* 1980).

In the parchment-shelled eggs of 11 species of lizards examined at the time of oviposition, albumen made up a mean of 3.2% of egg weight (range 1-7%), although in two additional species (*Amblyrhynchus cristatus* and *Conolophus subcristatus*) it was 19% and 28% respectively (Tracy and Snell 1985). These relatively low values, compared to crocodilian, turtle and bird eggs are difficult to interpret in isolation. Most lizards lay eggs at a late stage of embryonic development, when albumen could be expected to have been used. Furthermore, subembryonic fluid builds up within the oviducts, and may not necessarily need to be derived from albumen *per se*.

The slight difference in the relative proportions of albumen between *C. johnstoni* and *C. porosus* eggs are statistically significant (Table 1); *C. johnstoni* supply their ova (yolk mass) with significantly less albumen (0.92 grams albumen per gram of yolk) than do *C. porosus* (1.08) (non-overlap of 2SE's). *Alligator mississippiensis* is similar to *C. porosus* in providing more than 1 gram of albumen per gram of yolk.

Albumen density is 1.024 ± 0.001 g cm^{-3} for *C. johnstoni* and 1.020 ± 0.003 g cm^{-3} for *C. porosus*, which is higher than for the turtle *Carettochelys insculpta* (1.014 ± 0.004 g cm^{-3}; Webb *et al.* 1986a), but lower than for the domestic fowl (1.035 g cm^{-3}; Romanoff and Romanoff 1949).

Water is the main constituent of albumen (Table 3), with the remaining solid portion being almost entirely protein; no discernible quantities of lipid are present. Albumen of *C. johnstoni* has similar quantities of water (95.0%) to *C. porosus* (96.4%) and the turtle *Emydura macquarii* (95.4%; Thompson 1983), but more water and less protein than in the albumen of domestic fowl eggs (Table 3) and birds in general (85-90%; Ricklefs 1977). Yet surprisingly, the albumen of crocodilian and turtle eggs has a thicker consistency than that of birds, perhaps reflecting different types of proteins and chemical bonding between them.

EggShell

When considered as a percentage of total egg weight (Table 1), the shell and shell membrane of *C. johnstoni* (11.9 ± 0.3%) and *C. porosus* (12.0 ± 0.4%) are similar, whereas that of *A. mississippiensis* (12.9 ± 0.3%) is slightly heavier (significantly so when compared to *C. johnstoni*; non-overlap of 2SE's), and an even heavier shell and shell membrane (14.0%) may characterize *C. novaeguineae* eggs (Jenkins 1975; there is some doubt as to the reliability of the egg weights in this paper). All four species are within the range for turtle eggs with brittle shells (9.2-24.7%; Ewert 1979; Thompson 1983; Webb *et al.* 1986a), and are similar to precocial bird eggs (mean = 11.9%; Romanoff and Romanoff 1949), but are proportionally heavier than the shells of altricial bird eggs (mean = 7%; Romanoff and Romanoff 1949), and turtle eggs with parchment shells (4.3-11.0%; Tomita 1929; Simkiss 1962; Ewert 1979).

The eggshell membrane of crocodilian eggs is much thicker than that of bird eggs. In the domestic fowl the dry weight of the shell membrane comprises about 4% of the dry weight of the combined shell and membrane (calculated from Romanoff and Romanoff 1949) whereas in crocodilians it ranges from 6.8 to 21.4% (Table 4). In the absence of definitive data on the shell membrane in different species, variation in the percentage dry mass of the shell membrane within crocodilians may well indicate biological significance. The shell membrane of *A. mississippiensis* makes up the least mass (6.8%) [*Crocodylus johnstoni* (9.3%); *C. porosus* (12.7%) *Crocodylus acutus* (14.3%; calculated from Lutz *et al.* 1980)], and that of *C. novaeguineae* (21.4%; Jenkins 1975) the greatest.

High values for shell plus shell membrane could be explained by either a thick shell membrane relative to other species, or alternatively, a thin eggshell.

Table 4. The percentage water content of the shell and shell membrane of infertile crocodilian eggs in comparison to turtles and birds, and the percentage of the dry weight of the combined shell and shell membrane that is shell membrane.

Species	Percentage Water Content			Percentage Membrane Dry Weight
	Shell	Shell membrane	Shell + membrane	
Crocodilians				
A. mississippiensis[1]	2.9	46.4	8.4	6.8
C. acutus[2]	6.8	58.6	21.0	14.3
C. johnstoni	7.4	54.3	16.4	9.3
C. novaeguineae[3]	–	–	19.9	21.4
C. porosus	3.6	54.8	16.9	12.7
Chelonians				
Chrysemys picta[4]	–	–	26.9	–
Emydura macquarii[5]	–	–	22.3	–
Birds				
Gallus domesticus[6]	1.6	20.0	2.5	3.8

[1] data from M. W. J. Ferguson
[2] Lutz *et al.* 1980
[3] Jenkins 1975
[4] calculated from Lynn and von Brand 1945
[5] Thompson 1983
[6] Romanoff and Romanoff 1949

With regard to *C. novaeguineae*, Cox (1985) used eggshell thickness to discriminate between the eggs of that species (<0.483 mm) and those of *C. porosus* (>0.495 mm), which is consistent with *C. novaeguineae* having a relatively lighter shell, and not necessarily a heavier shell membrane. Preliminary investigations indicate that shell thicknesses in *C. porosus, C. johnstoni* and *A. mississippiensis* are similar, but the shell membrane of *A. mississippiensis* is thinner (Ferguson, pers. comm.), which is consistent with their low value (6.8%) on Table 4.

Water content of eggshells ranged from 2.9% in *A. mississippiensis* to 7.4% in *C. johnstoni* (Table 4). With respect to the shell membrane there was less interspecific variation in terms of water content, with *C. porosus, C. johnstoni* and *C. acutus* being similar to each other (54.3-58.6%) but higher than *A. mississippiensis* (46.4%). However, the data for *A. mississippiensis* (Table 4) are based on a single egg.

Differences in water content of the combined shell and shell membrane reflect the different water contents of the shell (Table 4). This may also explain in part the differences between crocodilians (8.4-21.0%) and the turtle *Chrysemys picta* (26.9%; calculated from Lynn and von Brand 1945), which has a parchment-shelled egg. Water content of the shell and shell membrane of *Emydura macquarii* (22.3%; Thompson 1983) is similar to that of both *C. acutus* and *C. novaeguineae*. Water content of the shell and membrane of domestic fowl eggs (2.5%) is less than that of all the reptiles for which there are data.

As a proportion of the total water content of eggs, the shell and shell membrane contribute little in all species. In *C. johnstoni* and *C. porosus* about 2.9% of total egg water is contained within the shell and shell membrane at or near the time of oviposition.

The structure of the shell of *A. mississippiensis* eggs has been studied in detail (Ferguson 1981a,b, 1982). It is composed of three main layers: an outer layer (0.1-0.2 mm); a 'honeycomb' layer (0.3-0.4 mm); and, a 'mammillary' layer (0.02-0.03 mm). The shell membrane is 0.15-0.25 mm thick. *Crocodylus acutus* shell thickness is 0.6 mm and the shell membrane thickness is 0.43 mm (Lutz *et al.* 1980).

Uptake of calcium from the shell and shell membrane (see "Calcium") may help weaken these protective layers as incubation proceeds in all species. Microbial degradation of *A. mississippiensis* egg shells enhances the ability of full-term embryos of this species to hatch spontaneously (Ferguson 1981a,b, pers. comm.), yet no such weakening is necessary for spontaneous hatching of either *C. porosus* or *C. johnstoni*. *Alligator mississippiensis* also hatch when the embryos are at a "young" stage relative to *Crocodylus* embryos (Ferguson 1985) and have a thinner shell membrane (Ferguson, pers. comm.). These differences are perhaps all related to *A. mississippiensis* embryos needing to develop rapidly and hatch during the relatively short period of the year when ambient temperatures allow successful development.

With regard to the ease with which crocodilian hatchlings escape from eggs when they are fully developed, it should be recognized that the shell membrane appears to form a greater barrier than the shell itself. Quite commonly hatchlings successfully "pip" the shell by pushing the caruncle against the shell membrane, but have great difficulty cutting through the membrane itself. It is not unusual to find rows of unsuccessful cuts on the inside of the shell membrane, and sometimes these are within eggs where parts of the shell have flaked away, yet the

embryo has died. In contrast, opened shell membranes within an egg with an intact shell have never been observed during the autopsy of full-term dead embryos. To this can be added the observation that in wet *C. porosus* nests, where eggs are swollen, cracked and stained, the shell membrane becomes noticeably weakened and is easily ruptured during handling.

Water Content of Eggs

Water holds the chemical constituents of the egg in solution and makes possible the various chemical activities that take place throughout development (Romanoff and Romanoff 1949). The total water content of infertile *C. johnstoni* and *C. porosus* eggs is 68.7% and 70.0% of total egg weight respectively. This is within the range for freshwater turtle eggs (66-72%; Cunningham and Hurwitz 1936; Ricklefs and Burger 1977; G. C. Packard and M. J. Packard 1980; Morris *et al.* 1983; Long 1985) and is similar to the total water content of domestic fowl eggs (66%; Romanoff and Romanoff 1949). However, the eggs of lizards (40-68% in 21 species; Vitt 1974, 1978; M. J. Packard *et al.* 1985) and snakes (39 to 69% in five species; Vitt 1978; M. J. Packard *et al.* 1984a, 1985) contain less water.

Differences in total water content between species are confounded by differences in shell size and type. If only the egg contents are taken into consideration, the water contents of *C. johnstoni* and *C. porosus* eggs are 75.7% and 77.2% respectively. Although *C. johnstoni* eggs contain relatively less water in the albumen than do *C. porosus* eggs (Table 3), the relatively larger yolk (Table 1), with a higher water content (Table 3) results in both species having proportionally similar total water supplies at the time of laying. Similar values have been obtained for *Chelydra serpentina* egg contents (79% water; Lynn and von Brand 1945; von Brand and Lynn 1947) and for the egg contents of 12 other species of turtles (61.2-72.8%; Congdon and Gibbons 1985). These values are somewhat intermediate between those of the egg contents of altricial (mean = 84.3%) and precocial (mean = 74.7%) birds (Carey *et al.* 1980).

It is perhaps not surprising that crocodile eggs are more 'hydrated' than lizard or snake eggs at the time of laying. Most squamate eggs are parchment-shelled and absorb water from the environment after laying. In addition, the late stage of embryological development at which most species lay their eggs means that some embryological water requirements could have been provided from sources within the maternal oviducts. The eggs of crocodilians and turtles are laid at an early stage of embryonic development (Ewert 1979, 1985; Ferguson 1985), and in at least brittle-shelled turtle eggs, total embryonic water supplies are provided from the start of incubation.

Water Losses and Gains

The eggs of both *C. porosus* and *C. johnstoni* are able to absorb water from a moist environment, but it is by no means an essential requisite for apparently normal development. In fact, it is usually a sign of conditions that are "too wet" in both wild nests and laboratory incubators, and is associated with high rates of embryonic mortality. Wild *C. porosus* nests, which are made in the wet season, often contain swollen eggs, and drowning of embryos is the major cause of mortality (Webb *et al.* 1977, 1983b). The nests of *C. johnstoni* are made during the dry season (Webb *et al.* 1983a), and up until the time of hatching rarely contain swollen eggs (although this can change overnight if heavy wet season rains occur early).

The process by which eggs do swell is unknown (Grigg and Beard 1985), but in *C. porosus* eggs, it occurs most commonly after about 45-50 days incubation (Grigg and Beard 1985; unpublished observations) when the chorioallantois surrounds the inside of the shell membrane (see Webb *et al.* Chapter 43). Simple diffusion driven by the ionic concentrations (osmolality) of the allantoic fluid (see later), would seem to be involved. Eggs that are completely inundated immediately after laying, or eggs which are infertile, rarely if ever swell. Thus incubation conditions that are too moist are not readily detected within the first few weeks of incubation. However, if swelling does occur, it can be reversed quickly by decreasing the humidity within incubators, although longitudinal cracks in the shell remain.

Under the incubation conditions used in this study, all eggs lost weight by the time they were opened or hatched. Mean water losses to hatching in eggs without air spaces ranged between 2.5% and 3.5% of initial weight. Large air spaces developed in some eggs, and, as mentioned earlier, they were interpreted as indicating higher than normal water losses. Such spaces have been considered an abnormal condition (Ferguson 1982), and if they are of gross proportions in either wild nests or laboratory incubators (>20-30% weight loss) they lead to stunting, high incidences of spinal abnormalities, failure to internalize residual yolk and generally high mortalities. However, like mild swelling, mild dehydration (<15% weight loss) does not appear to compromise embryo viability.

Eggs with air spaces occurred at all incubation temperatures we used other than 34°C. In 75% of eggs with evident air spaces (N = 59), the chorioallantois had parted from the eggshell membrane (Fig. 1c), and in 25% of cases, the eggshell membrane had parted from the shell (Fig. 1b). There is also a definite clutch effect on air spaces. For example, eggs from clutch 10 (30°C) had very porous shells and almost all developed extensive air spaces (up to 11.5% weight loss after 80 days

incubation). Analysis of the overall percentage of eggs with air spaces is confounded by some being opened before spaces may have developed and others having hatched rather than been opened, but around 24% of eggs probably had at least an easily discernible air space.

To examine generally the relationship between air spaces and weight loss in our eggs, individual eggs with air spaces were matched to eggs of the same age (± 2 days) being incubated at the same temperature, which did not have obvious air spaces. Eggs with air spaces averaged 2.7 ± 1.5 times the weight loss (as a proportion of initial egg weight) of eggs without air spaces (N = 32; range = 0.68-5.6). In two pairs of eggs (6.3%) weight loss was lower in the eggs with air spaces, and in one pair (3.1%), the weight loss was the same in both eggs. The extent of weight loss in the pairs of eggs was independent of real incubation time ($r^2 = 0.005$; $p>0.50$), or of standardized morphological age (MA_{30}) ($r^2 = 0.002$; $p>0.50$). Similarly, the different types of air spaces (Fig. 1b, c) were randomly distributed with respect to age.

Grigg and Beard (1985) found that rate of water loss was not related to incubation time in *C. porosus* eggs, which was in contrast to our findings and those of Whitehead (1987) with weight loss in *C. johnstoni* eggs. Weight losses generally accelerated after the chorioallantois had spread around the complete inner surface of the shell membrane (60 days MA_{30}), regardless of air spaces. Similarly, Ewert (1985) found increasing rates of water loss from chelonian eggs after late in incubation.

Embryo Size

The change in wet weight of *C. johnstoni* embryos with increasing MA_{30} is summarized in Figure 2. Only after 60 days of age at 30°C (MA_{30} = 60 days) is embryo size dependent upon egg size to a great degree (Webb *et al.* Chapter 50). Variation in embryo weight after 60 days of age (see Fig. 2), even after standardizing the data to the mean sized egg (68.2 g) is due to a number of other factors. For example differences in shell gas conductance between different eggs will affect growth and introduce variation that can not easily be accounted for. No relationship between embryo mass and the presence or absence of air spaces could be demonstrated.

Water contents of embryos in this study were not determined as they were required for another study (see Webb *et al.* Chapter 50). However, Whitehead (1987), using the same incubators and incubation technique recorded mean water contents for *C. johnstoni* hatchlings of 71.2% (with internal yolk included) and 77.7% (yolk-free). The value of 71.2% is similar to comparable data from chelonians (72-79%; Lynn and von Brand 1945; Ewert 1979; Kraemer and Bennett 1981; Thompson 1983), lizards

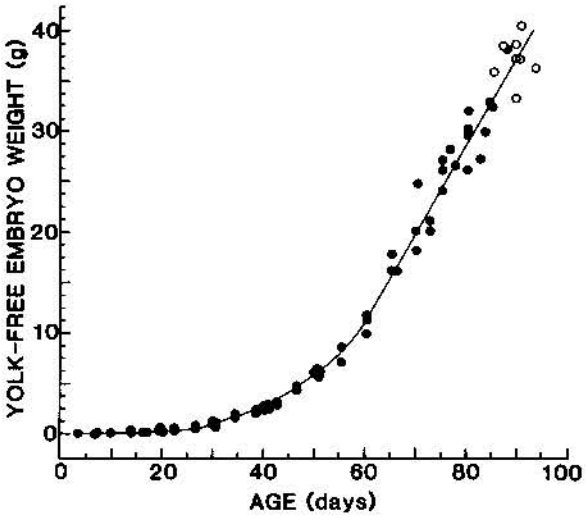


Fig. 2. Wet weight of *Crocodylus johnstoni* embryos as a function of days of incubation at 30°C. Weights after 60 days (see text) were standardized to the mean egg weight (68.2 g). Open circles indicate hatchlings. Line is eye-fitted.

(72-78%; Ewert 1979) and precocial birds (mean = 72.2%; Carey *et al.* 1980; Rahn 1984), but is lower than that from altricial birds (mean = 83.8%; Carey *et al.* 1980; Rahn 1984). Water content of newly hatched *C. porosus* was determined by Garnett (1983) to be 76.8%, but it is not known whether this refers to yolk-free weight or not. This investigator also determined that protein was the main constituent of the animal's dry mass — 62.3% protein (14.4% of wet weight), 17.5% fat (4.1% of wet weight) and 10.7% ash (2.5% of wet weight).

Yolk

Wet Weight of Yolk

The pattern of decrease in the wet weight of the yolk sac contents (excluding subembryonic fluid) was the same at all temperatures (Fig. 3). During the first 40 days MA_{30} the demands of the embryo are small, but so is embryo weight (Fig. 2). After 40-50 days MA_{30} the rate of yolk utilization increases markedly, which is partly due to a decrease in the water content of yolk (Fig. 4a), but mostly due to a decrease in yolk solids (Fig. 4b). It of course corresponds to the period of rapid embryo growth (Fig. 2).

In the turtles *Chrysemys picta* and *Chelydra serpentina*, a rapid decrease in the amount of solids in the yolk also occurs after about 50% of the way through incubation (Morris *et al.* 1983; G. C. Packard *et al.* 1983; G. C. Packard and M. J. Packard 1984).

The total amount of yolk used (relative to the amount present at the time of oviposition) up until the time of hatching, varies with incubation temperature (Table 5). Animals incubated at low temperatures use a greater proportion of their total yolk supplies during their extended period in the egg.

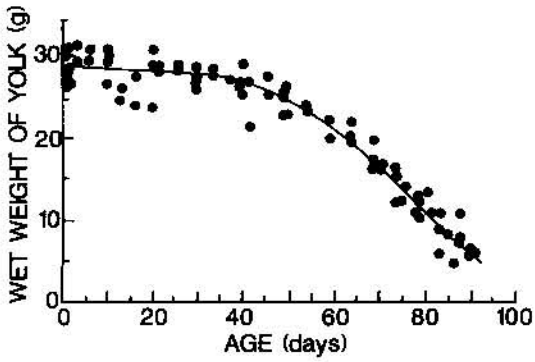


Fig. 3. Wet weight of yolk in *Crocodylus johnstoni* eggs as a function of days of incubation at 30°C. Weights were standardized to the mean egg weight (68.2 g).

Table 5. The effect of incubation temperature on the proportion of initial yolk mass (wet weight) utilized by *Crocodylus johnstoni* embryos up until the time of hatching. SE = standard error.

Temperature °C	% of initial yolk used	SE	N
28	87.1	1.3	6
29	83.4	1.5	9
30	78.5	1.3	6
31	71.0	1.6	7
32	60.5	3.2	10
33	71.2	1.5	8
34	63.6	1.6	5

This is not simply an increased maintenance cost, as the slower developing animals hatch when they are larger and at a more advanced stage of development (see Webb *et al.* Chapter 50).

Yolk Water

The yolk of crocodilian (Ferguson 1985), turtle (Morris *et al.* 1983; G. C. Packard *et al.* 1983) and bird (Romanoff and Romanoff 1949; Romanoff 1967) eggs becomes more viscous as development proceeds. With *C. johnstoni* eggs (Fig. 4a), the water content of yolk is highly predictable from morphological age ($r^2 = 0.81, p<<0.001$), independent of temperature. It decreases from about 58% at the time of laying (Table 3) to 49% after 85 days MA_{30}.

At hatching, the water content of *C. johnstoni* yolk (about 48%) is similar to the water content of *Caretta caretta* yolk (44.9%; Kraemer and Bennett 1981) but is less than that in the snake *Coluber constrictor* (60%; M. J. Packard *et al.* 1984a) and more than that in domestic fowls (about 30%; Romanoff 1967).

No effects of dehydration (egg weight loss) on yolk water content could be demonstrated, suggesting that most (if not all) of the water losses come from compartments other than the yolk, as found in domestic fowl eggs incubated under different humidity regimes (Hoyt 1979). In *Chrysemys picta* the rate of water loss from yolk was the same after 30 days of incubation for eggs incubated under different hydric environments (G. C. Packard *et al.*

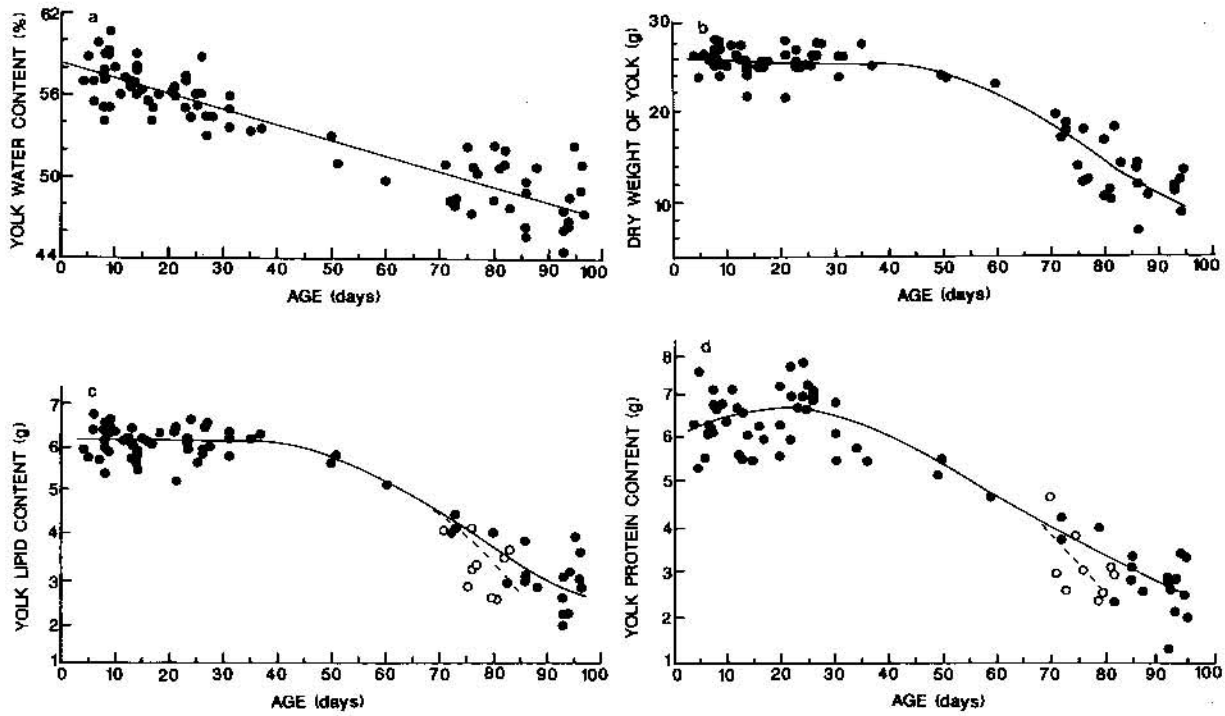


Fig. 4. Water content *(a)*, dry weight *(b)*, lipid content *(c)*, and protein content *(d)* of *Crocodylus johnstoni* yolk throughout incubation. Lines (on *a,b,c*) are eye-fitted and open circles (and dashed lines) distinguish eggs with air spaces. Data come from incubation temperatures between 28°C and 34°C, but are standardized to 30°C (MA_{30}) and to mean egg weight (68.2 g).

1983; G. C. Packard and M. J. Packard 1984), although Morris *et al.* (1983) found that hatchling *Chelydra serpentina* incubated under different moisture regimes differed in the water content of their residual yolk. This result may well be correct, however, if the incubation regimes affect yolk utilization and stage of development at hatching, as in crocodile eggs (Table 5), then such differences may be explicable on the basis of the effects of those incubation regimes on the relative time of hatching.

Yolk Lipids and Proteins

Lipids contain more energy per gram than proteins [39.5 kJ g^{-1} for lipid; 23.6 kJ g^{-1} for protein (Brody 1945)], and more water is produced as a by-product of their combustion (per gram) than comes from protein (Needham 1963). However, when embryos utilize proteins for energy, they can only extract about 77% of the potential energy (as measured by calorimetry), whereas they can extract about 95% of the potential energy of lipids (Ewert 1979). Furthermore, protein catabolism produces substantial amounts of nitrogenous wastes that the embryo must accommodate in one form or another, all of which require energy. Accordingly, the relative amounts of lipid and protein in the egg are potentially important considerations in furthering our understanding of embryonic metabolism (Ewert 1979).

About 83% of the total protein stores in *C. johnstoni* eggs are within the yolk, which implies a heavy reliance on protein metabolism relative to what occurs in birds (Ewert 1979) (although the benefits of this to an embryo are unknown). Yet when compared to other reptiles, the relatively high lipid content of *C. johnstoni* eggs implies a greater dependance on lipid metabolism than occurs in other reptiles. The difficulty of course lies in resolving just how much of the protein and lipid stores, from both albumen and yolk, are actually used for the production of energy.

The average rate of lipid utilization in *C. johnstoni* up to 50 days MA_{30} is 0.7 mg d^{-1}, increasing to an average rate of 70.0 mg d^{-1} after 50 days MA_{30} (Fig. 4c); protein utilization proceeds in a similar fashion, albeit at a higher rate [3.4 mg d^{-1} up to 50 days MA_{30}; 80.7 mg d^{-1} after 50 days MA_{30} (Fig. 4d)]. The slight increase in the amount of protein within the yolk sac in the early part of incubation (Fig. 4d) results from albumen protein entering the yolk sac with the water that forms subembryonic fluid (see later).

These results are consistent with protein rather than lipid being used as the preferred substrate for biosynthesis during the early part of incubation, as in *A. mississippiensis* (Clark *et al.* 1957). Crocodilian embryos are making a substantial commitment to extraembryonic membranes at the time of egg-laying. This same structural commitment occurs relatively later in birds.

In domestic fowl embryos, yolk lipids disappear sharply after about 70% of incubation (Romanoff 1967), which is relatively later than appears to be the case in *C. johnstoni*. The rapid increase in the rate of utilization of both lipid and protein (Fig. 4c, d) occurs when the embryo begins to grow rapidly (Fig. 2) (Webb *et al.* 1986b; Whitehead 1987); after the respiratory 'organ', the chorioallantois, fully covers the inner surface of the shell membrane.

About 57% of the total lipid content of the egg and 63% of all protein is used by the time of hatching at 30°C. If the proportions of lipid and protein in the yolk are indicative of proportional utilization of energy sources, crocodilians (like other reptiles) rely more on protein than do birds, although crocodilians themselves use more lipid than do turtles (Lynn and von Brand 1945; Chaikoff and Entenman 1946; von Brand and Lynn 1947; Needham 1963; Ricklefs and Burger 1977; Ewert 1979; Congdon *et al.* 1983; Thompson 1983), and may thus be closer to the birds in this respect.

Effects of Water Loss on Lipid and Protein Use

Eggs with air spaces (open circles on Fig. 4c, d) show different patterns of lipid and protein utilization than do eggs without air spaces during the final stages of incubation. Domestic fowl embryos with low porosity shells exhibit reduced metabolism (Tullett and Deeming 1982), and are able to regulate their development according to the availability of nutrients (Simkiss 1980). *Crocodylus johnstoni* embryos are able to enhance the internalization of yolk and hatch early if incubation temperatures are high (Webb *et al.* Chapter 50). The data on Figures 4c and 4d suggest that dehydration may stimulate the development rate of the embryo in a fashion similar to high temperature; namely, enhancing the internalization of yolk and bringing forward the time of hatching.

Clearly, there is a great deal to learn about how the homeostatic mechanisms of embryos adjust to increasing rates of water loss. The effects of humidity on the rate of development of crocodilian embryos is a step in the right direction in helping understand this complex interaction.

Albumen

Wet Weight of Albumen

When standardized to mean egg size and 30°C morphological ages, the pattern of albumen utilization over time (Fig. 5) could be subdivided into four distinct phases. Using the 30°C results as a standard, these are:

1. An initial phase over the first 10 days MA_{30} characterized by the highest rate of albumen disappearence. Water and protein are transferred into the

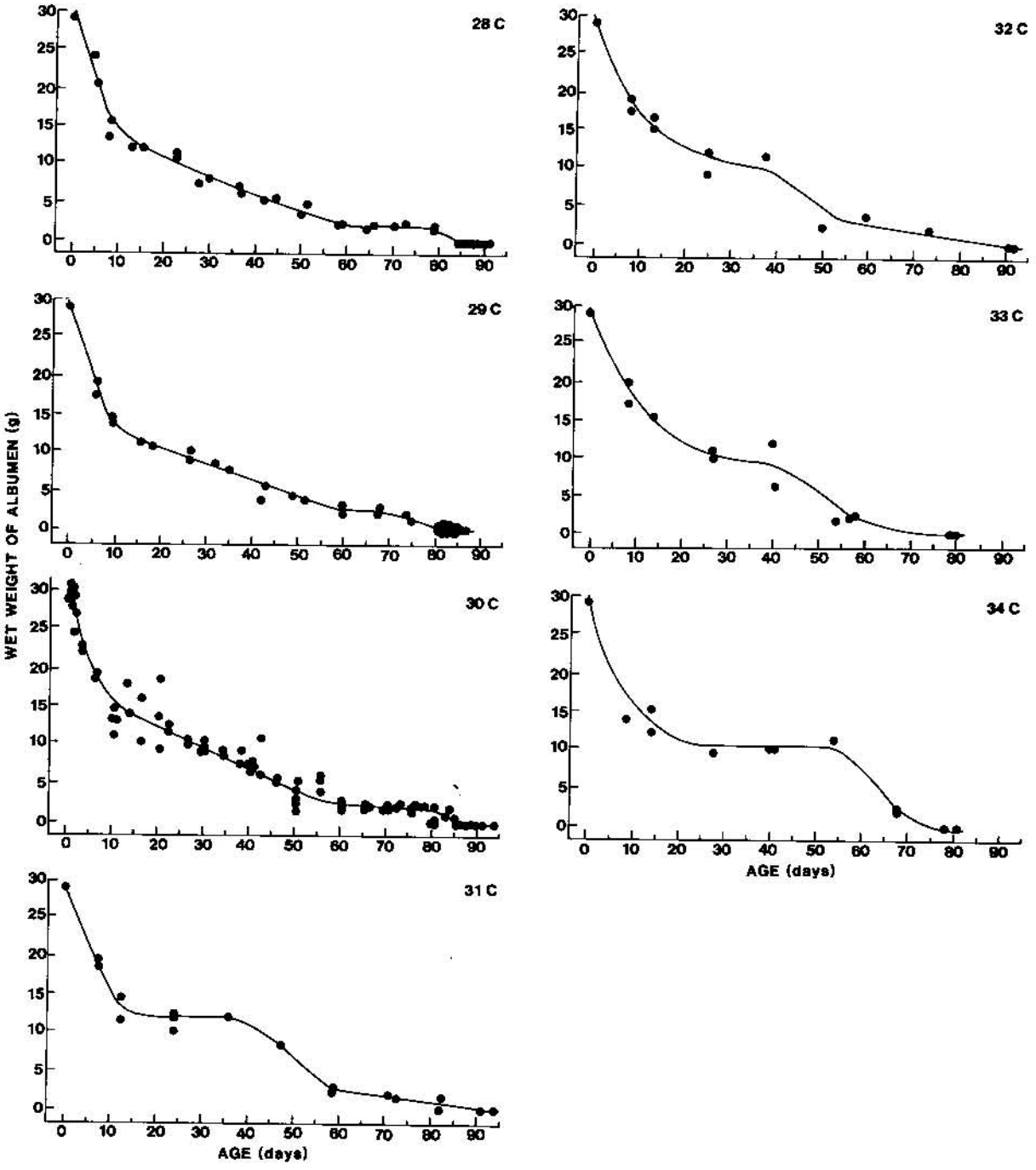


Fig. 5. Wet weight of albumen in *Crocodylus johnstoni* eggs incubated at various temperatures. Data are standardized to mean egg weight (68.2 g) and to 30°C rates of development (MA_{30}). Lines are eye-fitted.

yolk sac to form subembryonic fluid. At low incubation temperatures a greater proportion of the initial albumen stores are used than at high incubation temperatures (when scaled to MA_{30}). By the end of the first phase, 55-60% of initial albumen weight has been utilized.

2. The second phase (10-55 days MA_{30}) is characterized by a reduced rate of albumen utilization, and corresponds to the time when the chorioallantois is spreading over the inside of the shell membrane. At 28°C, 29°C and 30°C this phase scales to the same MA_{30} days, but at higher temperatures the weight of albumen changes little until about 40 days MA_{30} at 31°C, 32°C and 33°C, and at 55 days MA_{30} at 34°C.
3. In the third phase (55-80 days MA_{30}), there is a negligible rate of decrease at all temperatures (although there are few data points at the higher temperatures). About 90% of all albumen has been used by 60 days MA_{30} and the chorioallantois has completely spread over the inner surface of the shell membrane.

4. The first eggs with no albumen remaining appeared at 78 days MA_{30} (at 34°C) and most eggs could be expected to have none by about 80 days MA_{30}. The space originally occupied by the albumen is now filled with the embryo, yolk and allantoic sac.

Albumen Water

The relative water content of albumen decreases with time (Whitehead 1987). After 35 days MA_{30}, about 70% of the initial albumen weight has disappeared, and the water content of the remaining albumen has decreased from 95% (at time of laying) to about 88%. Accordingly, the protein portion accounts for a greater proportion of the remaining albumen weight. As water is removed, crocodilian albumen becomes rubbery in consistency, as in turtles (Morris *et al.* 1983; G. C. Packard *et al.* 1983). By three-quarters of incubation the water content of any remaining albumen is around 55%, after which time it increases to about 70% (Whitehead 1987).

Subembryonic Fluid

At the time of laying, in fertile eggs, a small amount of fluid is within the yolk sac immediately under the embryonic disc (Webb *et al.* 1986b). The bulk of this subembryonic fluid is water which has been drawn from the adjacent albumen, as in birds (Romanoff 1967) and turtles (Ewert 1979, 1985).

A number of possible functions have been assigned to subembryonic fluid: a reservoir for excretory products before the allantois is formed (Clark *et al.* 1957; Ferguson 1985); a supply of water for the allantois when it is formed (Romanoff 1967); and, a means of enhancing respiration as the fluid distends the vitelline sac and pushes the embryo closer to the shell membrane (New 1956). More recently (see Webb *et al.* 1986b, Chapter 43), the low density of the pocket of subembryonic fluid (relative to yolk density) has been implicated in the mechanism by which the yolk rotates after laying, bringing the embryo to the upper surface of the egg.

Subembryonic fluid does not appear in infertile eggs, regardless of how long they are kept at any incubation temperature. In contrast, apparently infertile eggs (no opaque band) sometimes have a small dead embryo on the vitelline membrane, and a small pocket of subembryonic fluid. The presence of a live embryo appears essential for significant amounts of water to diffuse from the albumen into the vitelline sac contents. New (1956) demonstrated that fluid was absorbed by the ectodermal surfaces of the blastoderm and secreted from its endodermal surfaces into the vitelline sac. The site of the embryo is the major site for both albumen dehydration and subembryonic fluid formation in eggs with young embryos.

Later, the dehydration of albumen may be effected by osmotic forces between the albumen and the subembryonic fluid (Needham 1963). The albumen of bird eggs is markedly hyperosmotic to yolk (Potts and Parry 1964). However, an active role is certainly played by the embryo in crocodilian eggs, as subembryonic fluid production ceases with embryonic death.

The volume of subembryonic fluid increases rapidly after laying (Fig. 6) and at 30°C maximum volume (18-20 cm^3 in an average sized egg) is reached after about 13-14 days of age. After this time subembryonic fluid volume decreases, until no fluid remains (at about 46 days). The same pattern is evident in *C. porosus* eggs incubated at 30°C (Webb *et al.* 1986b, Chapter 43).

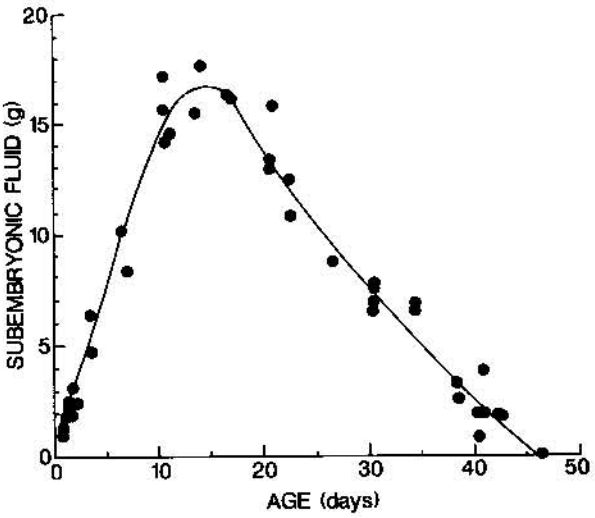


Fig. 6. Wet weight of subembryonic fluid in *Crocodylus johnstoni* eggs incubated at 30°C. Data are standardized to mean egg weight (68.2 g) and volume (in cm^3) can be calculated by multiplying fluid weight by 0.995.

In both species, maximum volume occurs after about 15% of incubation time, which is markedly less than the 30% reported for avian eggs (Romanoff 1967). Similar data are not available for turtles, and although Morris *et al.* (1983) describe changes in the weight of the yolk sac contents for *Chelydra serpentina*, they do not separate those contents into major component parts. Assuming that the peak in weight of yolk contents corresponds to the time of maximum volume of subembryonic fluid, then it would occur after about 35% of incubation time (Morris *et al.* 1983).

Within our data, many more eggs were examined at 30°C than at the other temperatures, and so some extrapolation was used to determine approximately the times at which maximum subembryonic fluid volume occurs, and when it disappears completely (Table 6). Maximum volume occurs at a similar MA_{30}, regardless of the temperature of incubation (Table 6). However, the total volume accumulated varies

Table 6. The real ages (RA) and standardized morphological ages (MA_{30}) in days, at which the maximum volume of subembryonic fluid occurred, and at which subembryonic fluid ceased to exist (zero volume), in *Crocodylus johnstoni* eggs incubated at different temperatures.

Temperature	Maximum Volume		Zero Volume	
°C	RA	MA	RA	MA
28	19	14	65	47
29	15	13	55	46
30	13	13	46	46
31	12	14	39	46
32	12	14	39	48
33	10	13	35	46
34	10	13	38	51

with temperature; about 19 cm^3 in an average sized egg at 28-30°C, and 15-17 cm^3 at 31-34°C. These differences are reflected directly in the amount of albumen used at different temperatures (about 1 gram of subembryonic fluid is formed from 1 gram of albumen).

The stage at which no subembryonic fluid remained was similar at all temperatures except 34°C (Table 6), where it remained relatively longer. This may be an artefact of small sample sizes, but could easily be a real temperature effect. At 34°C development is greatly enhanced, abnormalities are common and embryo survivorship is low (Webb *et al.* 1983a). In the avian egg subembryonic fluid is present over 65% of the incubation period (Romanoff 1967) compared to about 50% in *C. johnstoni* and *C. porosus* (Webb *et al.* 1986b, Chapter 43).

The mean density of subembryonic fluid between 10 and 30 days (at 30°C), in samples where we were confident that little or no mixing with yolk had occurred, did not vary with embryo age: *C. johnstoni*, 1.005 ± 0.001 g cm^{-3} (N = 11); *C. porosus*, 1.004 ± 0.002 g cm^{-3} (N = 5). In the freshwater turtle *Carettochelys insculpta* the density of subembryonic fluid was higher (1.01 g cm^{-3}; Webb *et al.* 1986a). In *C. porosus* eggs subembryonic fluid removed near the time of maximum volume was found to have a mean water content of 98.8 ± 0.1% (N = 3).

Ion Composition of Subembryonic Fluid

The range of times over which ionic composition was determined did not cover the first 12 days of incubation (Table 7).

Sodium and chloride are by far the ions in highest concentrations, and the amounts of both of these, as well as of calcium, creatinine, inorganic phosphate and bicarbonate, decrease as the volume of subembryonic fluid decreases. This trend is similar to that in the eggs of domestic fowls (Romanoff 1967), and suggests that the ionic composition of subembryonic fluid (or changes in it) could effect the transfer of water from albumen by simple diffusion.

At the time when subembryonic fluid volume is being reduced, through movement to the expanding amnion and allantois, the amounts of the major ions within the subembryonic fluid are also reduced (Table 7). This correlation suggests that the water may passively follow as ions are actively removed, as appears to be the case with allantoic fluid. Whether ion pumps are present in the vitelline membrane or yolk sac membrane is unknown, but if so, sodium and chloride ions are perhaps the major ones involved.

Table 7. Ionic composition of subembryonic fluid from *Crocodylus johnstoni* eggs. Data has been standardized to mean egg size (68.2 g) and results from incubation temperatures other than 30°C have been standardized to morphological ages (MA_{30}). Values are means of five day units of MA_{30}; mM = mmoles l^{-1}.

	Bicarbonate		Calcium		Chloride		Sodium	
Days	mM	mmoles	mM	mmoles ($\times 10^{-2}$)	mM	mmoles	mM	mmoles
10-15	14.0	0.22	1.26	1.97	95	1.56	110	1.73
15-20	11.0	0.17	1.06	1.65	99	1.47	112	1.69
20-25	10.0	0.13	1.04	1.32	91	1.15	85	1.05
25-30	10.0	0.10	1.31	1.28	89	0.88	94	0.93
30-35	11.2	0.08	1.62	1.17	82	0.61	85	0.63
35-40	17.0	0.09	1.53	0.84	86	0.46	89	0.50
40-45	26.0	0.15	1.62	0.92	93	0.52	100	0.58

	Urea		Urate		Creatinine		Inorganic phosphate	
Days	mM	mmoles ($\times 10^{-2}$)	mM	mmoles ($\times 10^{-4}$)	mM	mmoles ($\times 10^{-3}$)	mM	mmoles ($\times 10^{-3}$)
10-15	0.50	0.78	0.000	0.00	0.09	1.40	0.50	0.78
15-20	0.55	0.85	0.000	0.00	0.06	0.92	0.55	0.84
20-25	1.00	1.25	0.010	1.24	0.07	0.87	0.57	0.72
25-30	1.20	1.15	0.007	0.69	0.09	0.87	0.73	0.70
30-35	1.30	0.95	0.010	0.74	0.10	0.73	0.86	0.63
35-40	1.40	0.77	0.010	0.55	0.11	0.60	0.72	0.40
40-45	1.90	1.08	0.010	0.56	0.13	0.74	0.22	0.12

The amounts of urea and soluble urates in subembryonic fluid were minor, but increased slightly with time; as there is a concurrent decreasing volume, it indicates increasing concentrations. In the eggs of domestic fowls, uric acid is the main excretory product and is in much higher concentrations than urea (Romanoff 1967). In *C. johnstoni*, urea is the main nitrogenous waste (see later). Since subembryonic fluid probably acts as a reservoir for these excretory products until the allantois is formed (Clark *et al.* 1957; Ferguson 1985), and the embryo is still very small at the time it is formed (Ferguson 1985; see later), it is not surprising that urea and urates are both present and in low quantities (and therefore low concentrations).

Allantoic Fluid

Allantoic Membrane

The allantois begins to form at about 4 days MA_{30} (Stage 4; Ferguson 1985) and by nine days MA_{30} is a small, clear, fluid-filled sphere which makes contact with the chorion (Stage 9; Ferguson 1985) forming the chorioallantois. This stage is usually considered sensitive, in that mechanical shock may rupture one or both of the extraembryonic membranes, resulting in embryo mortality (Ferguson, pers. comm.). From here, the allantoic sac steadily enlarges, and the fusion with the chorion spreads around the inside of the opaque area of the shell. The detailed histological structure and disposition of the fused chorioallantoic membranes versus unfused allantoic membranes remains unclear, although the allantoic or chorioallantoic sac can be readily discerned and removed.

The chorioallantoic sac increases to an average wet weight of about 3 g at all temperatures, around 60 days MA_{30} (Fig. 7a), but decreases to about 1.5 g by the time of hatching. This decrease also occurs in avian eggs (Romanoff 1967), although the reasons for it are unknown. It may be due to dehydration of the chorion, which is attached to the shell membrane, or it may be related to structural changes in the membrane itself. That the dry weight of the chorioallantoic membrane in *C. johnstoni* also decreases during the latter stages of incubation (Whitehead 1987), suggests structural changes.

Relationship between the Chorioallantois and Opaque Banding

The growth of the chorioallantois is intimately linked with opaque band development (Webb *et al.* 1986b, Chapter 43). In general the following time periods (expressed as MA_{30} ages) are significant:

0-10 DAYS: Up to 24 hours after laying the egg is translucent, and only by candling (and detecting subembryonic fluid or a blastodisc) can fertile eggs be distinguished from infertile ones. The opaque band begins as a spot and expands around the sides of the egg: by two days it is 50% around the egg, and by three days 75%. The band is usually complete by four days, and has an even width by 10 days. This sequence is almost identical in *A. mississippiensis* (Ferguson 1982, 1985) and *C. porosus* (Webb *et al.* 1983b). The opacity is due to structural changes between the shell and shell membrane (Ferguson 1982), probably caused by the same mechanism dehydrating the albumen (Webb *et al.* Chapter 43). Where the shell is opaque, the vitelline membrane is against the shell membrane (the albumen originally between them has been dehydrated).

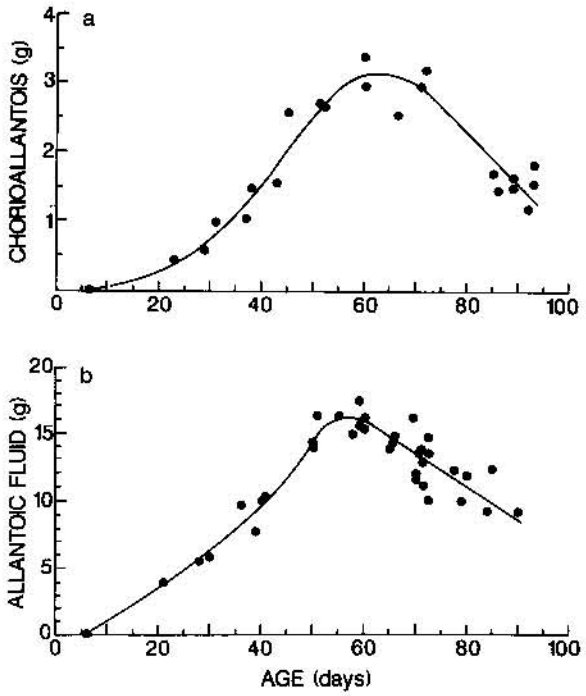


Fig. 7. Wet weight of the chorioallantois *(a)* and allantoic fluid *(b)* in *Crocodylus johnstoni* eggs incubated at 30°C. Data are standardized to mean egg weight (68.2 g).

10-35 DAYS: The opaque band remains stable throughout this period, but the chorioallantois develops within the opaque area. By 35 days the highly vascular chorioallantois is restricted to, but completely covers, the shell membrane within the opaque band. These events and their timing are similar in *C. porosus* (Cox 1985; Webb *et al.* 1986b), *C. novaeguineae* (Cox 1985) and *A. mississippiensis* (Ferguson 1982).

35-60 DAYS: Typically, the opacity begins to spread laterally from the band between 35-40 days MA_{30}, often moving towards one egg pole before the other. The expanding opacity mirrors the dehydration of albumen by the spreading chorioallantois.

60+DAYS: The egg is uniformly opaque, with the chorioallantois fully covering the inner surface of the shell membrane, and with little or no hydrated albumen in contact with the shell membrane. Although *C. porosus* and *C. novaeguineae* are similar to *C. johnstoni* in this respect (Cox 1985; Webb *et al.* 1986b), *A. mississippiensis* is not (Ferguson 1982); eggs are completely opaque by 45 days (Ferguson 1982). (This difference may relate to the shorter incubation time of *A. mississippiensis*). When expressed as a proportion of incubation time, complete opacity occurs 65-70% of the way through incubation in all four species.

Allantoic Fluid

The amount of allantoic fluid increases to a maximum volume at about 50-60 days of age (at 30°C) (Fig. 7b): 16 cm^3 in an average sized egg. By the time of hatching, volume is reduced to about 10 cm^3. In accordance with the effect of incubation temperature on the relative stage of yolk internalization and hatching (Webb *et al.* Chapter 50), hatchlings from low temperature incubation (delayed hatching) have less allantoic fluid remaining than those from high incubation temperatures (enhanced hatching). That there is any allantoic fluid remaining at hatching is in marked contrast to the situation in birds, where allantoic fluid is normally totally resorbed by 70% of incubation (Simkiss 1980).

The pattern of buildup of allantoic fluid, and the maximum volumes attained, were similar at all temperatures, suggesting they are intimately associated with the stage of embryo development rather than with incubation time or temperature. Eggs with air spaces generally had less allantoic fluid at a particular stage than did those without air spaces, which suggests that water loss is largely from the allantoic fluids next to the shell membrane, as occurs in domestic fowl eggs incubated under different humidity regimes (Hoyt 1979). Given the site of water loss and the amounts of water remaining in the allantois at the time of hatching, relatively high water losses may not necessarily affect embryo size at hatching, although extreme water losses are fatal.

Ion Composition of Allantoic Fluid

The ions in highest concentrations within allantoic fluid are sodium and chloride, and the concentrations of both decrease with time (Fig. 8). The total amounts of each of these ions increase up to 40-50 days MA_{30}, to reach 0.9 mmoles of sodium and 1.2 mmoles of chloride, after which there is a decline in the amounts present. The observed increase in chloride concentration after 70-75 days MA_{30} is therefore due to the decreasing volume of allantoic fluid, rather than more of the ion being present. However, there is no consistent effect of dehydration (as evidenced by air spaces) on the concentrations of either ion.

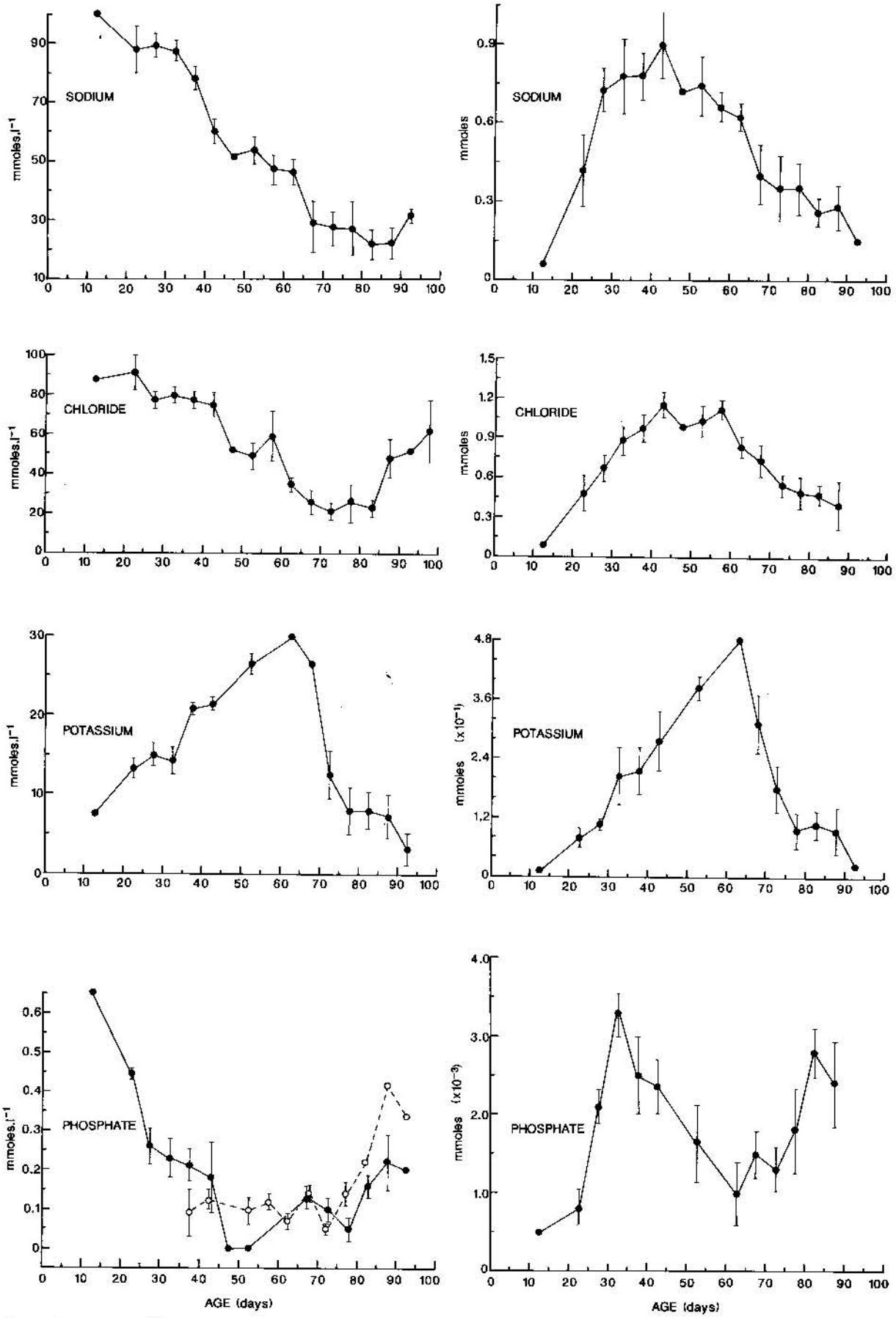
The amounts of potassium increase up to 60-65 days MA_{30} and then decrease, although as with sodium and chloride, there is no apparent effect of dehydration on concentrations. The concentration of inorganic phosphate rapidly decreases from about 0.65 mmoles l^{-1} at 10-15 days MA_{30} to less than 0.1 mmoles l^{-1} at 45-55 days MA_{30} (the zero values shown on Fig. 8 for this ion indicate low concentrations outside the range of the instrument used to measure them). However, when air spaces are present, the concentrations of inorganic phosphate are generally higher; concentrations appear to be determined by the volume of allantoic fluid at any given time. The amount of inorganic phosphate is relatively minor compared to the other ions (0.5×10^{-3} mmoles at 10-15 days MA_{30} to 2.8×10^{-3} mmoles after 85 days MA_{30}).

Bicarbonate concentrations follow a similar pattern to potassium, although there is a maximum concentration of 15 mmoles l^{-1} at 10-15 days MA_{30}. After a decline to 7.5 mmoles l^{-1} at 20-25 days MA_{30} there is a rise in concentration at 45-50 days MA_{30} (12 mmoles l^{-1}) and then a steady decline up until hatching. After 60-65 days MA_{30}, the amount of bicarbonate ion decreases in all eggs, though levels are much lower in eggs with air spaces until 80-85 days MA_{30}, when no such difference is apparent (Fig. 8).

The concentrations of calcium increase markedly after 50-55 days MA_{30}, and this is due to a marked increase in the amount of ion present. In dehydrated eggs concentrations were higher (see later: "Calcium").

There was a marked increase in the amount of urea in the allantois after 55-60 days MA_{30}, with creatinine following a similar pattern, albeit at much lower levels. The concentrations of both these ions increase with water loss from the allantois. Urate levels increase markedly at 55-60 days MA_{30} also, but after 70-75 days MA_{30} the amount of soluble urate in eggs with air spaces falls markedly, whereas this does not occur in 'normal' eggs until 80-85 days MA_{30}.

The osmolality of allantoic fluid decreases with increasing volume (Fig. 9), and increases as water is resorbed from the allantois after 60 days MA_{30}. This is in contrast to the situation in domestic fowl eggs, where the osmolality of allantoic fluid decreases throughout the last half of incubation (Romanoff 1967), and the difference almost certainly reflects the different proportions of the nitrogenous wastes



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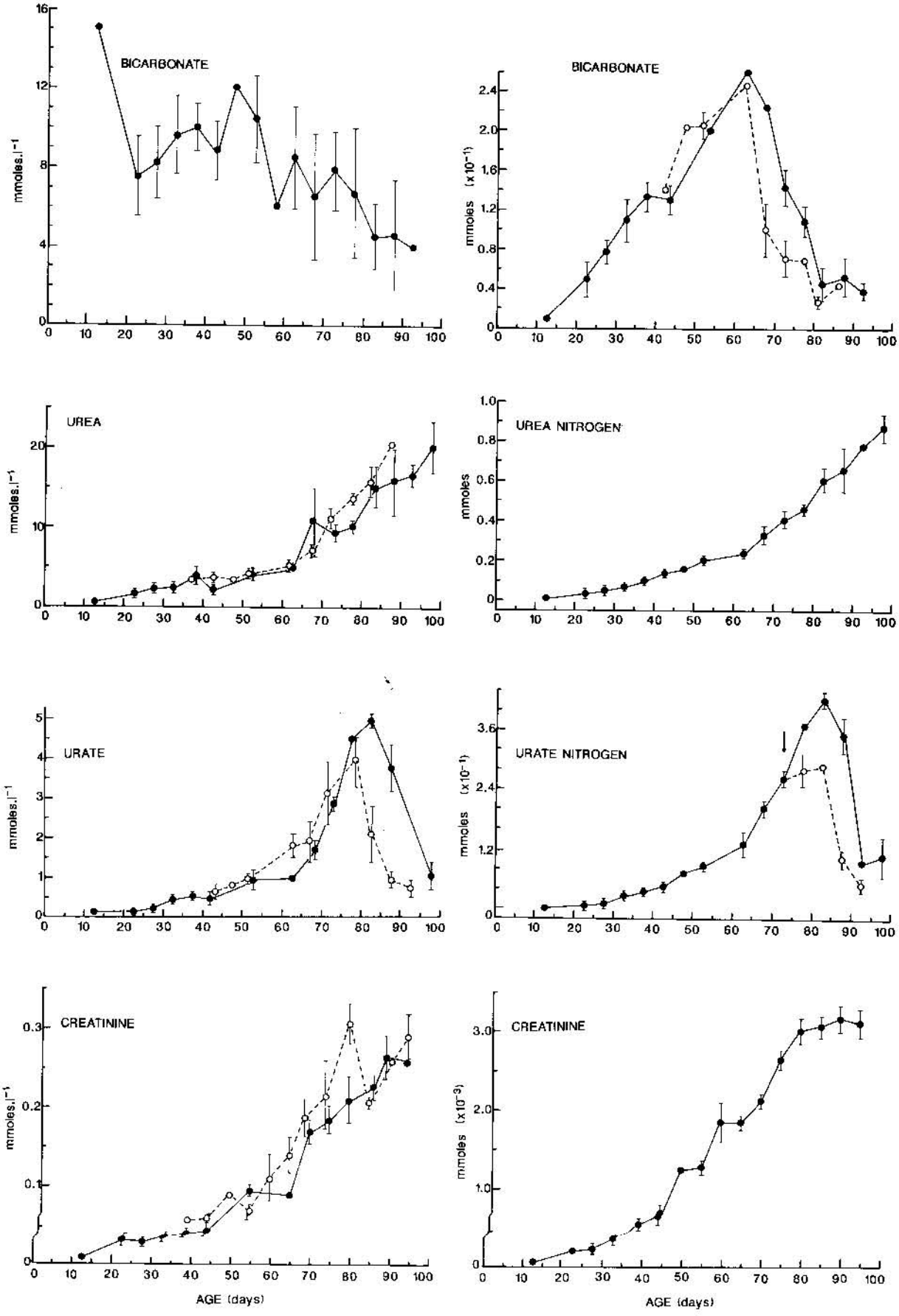

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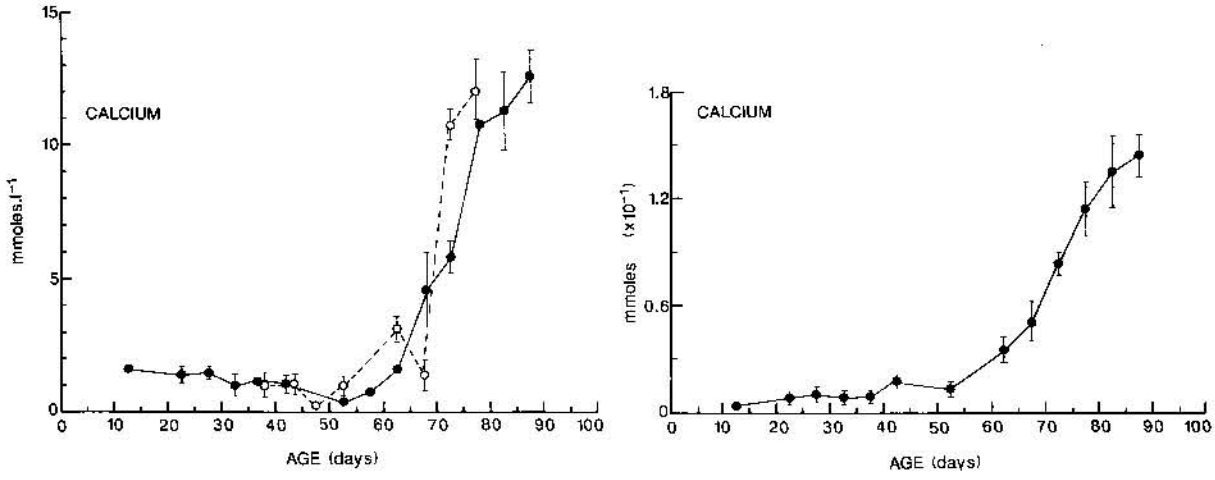


Fig. 8. The concentrations and relative amounts of the major ions in the allantoic fluid of *Crocodylus johnstoni* eggs. Data from incubation temperatures between 28°C and 34°C were standardized to 30°C rates of development (MA_{30}), and all weights were standardized to mean egg weight (68.2 g). Means and one standard error refer to five day intervals of MA_{30}. Open circles indicate eggs with air spaces, and closed circles eggs without air spaces. Where there are only closed circles, this indicates similar trends with and without air spaces, and all data were pooled.

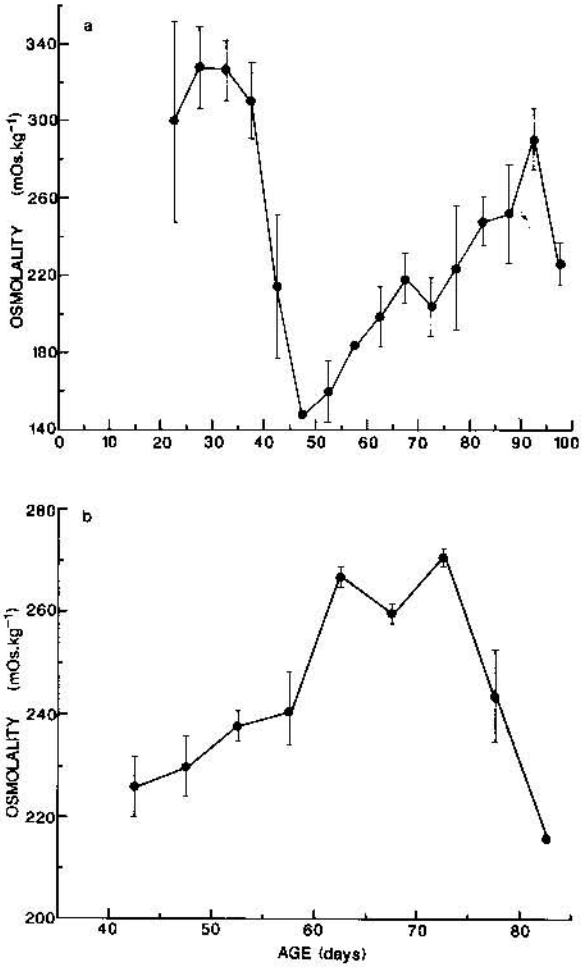


Fig. 9. The osmolality of allantoic *(a)* and chorio-amniotic *(b)* fluids of *Crocodylus johnstoni* eggs. Data from incubation temperatures between 28°C and 34°C are standardized to 30°C relative ages (MA_{30}) and are expressed as the means with one standard error for intervals of five days MA_{30}.

excreted: avian embryos excrete mainly insoluble uric acid with very little urea, whereas *C. johnstoni* excrete relatively large amounts of urea (see later).

That there were no significant differences between the osmolalities of allantoic fluid between eggs with and without air spaces is discussed below, but less bicarbonate and urate in solution in dehydrated eggs may help in reducing the overall ionic concentration, thereby reducing differences in osmolality between the two groups.

Water Resorption

The water of allantoic fluid is derived both from the yolk sac (from subembryonic fluid) and from dehydration of the remaining albumen. Increases in the amounts of the major ions (particularly sodium and chloride; Fig. 8) within the allantois probably reflect the transfer of water into the allantois from other compartments, just as when water is resorbed from the allantois, the amounts of sodium, chloride and potassium decrease.

In domestic fowl eggs, changes in the ultrastructure of the chorioallantois occur at the time fluid is resorbed from the allantois (Coleman and Terepka 1972), and these changes appear to facilitate the absorption of sodium and/or chloride against a gradient (Stewart and Terepka 1969; Moriarty and Hogben 1970). The endodermal surface of the allantois is thought to be involved in the resorption of these ions (Coleman and Terepka 1972) and the process is thought to be associated with Na-K ATPase in the granular cells of the allantois (Saleuddin *et al.* 1976).

Our data on the ionic composition of allantoic fluid in *C. johnstoni* eggs suggest that a similar

process is operating. The decrease in the dry weight of the chorioallantois in *C. johnstoni* eggs (Whitehead 1987) suggests that structural changes do occur in the membrane, and the removal of sodium and chloride ions from the allantoic fluid (Fig. 8), suggests active ion pumps within the chorioallantois. Whether sodium alone is actively transported and chloride follows passively or whether sodium and chloride are both actively transported is unknown. Similarly, potassium is also lost from allantoic fluid, but whether it is directly involved with water resorption is unknown. The nett result of the removal of ions from the allantois can reasonably be expected to be water following passively with them (Martin and Diamond 1966), entering the chorioallantoic membrane and thus the vascular system of the embryo.

Loss of significant quantities of water from the allantois through dehydration results in increased concentrations of calcium, inorganic phosphate, urea, creatinine and urate (up to 70-75 days), but not of sodium, chloride, potassium and bicarbonate (Fig. 8). At times of high water loss, the domestic fowl embryo regulates its body water by utilizing water from the allantois (Hoyt 1979), and control of such resorption must involve regulation of the major ions. We suspect this is also the case with *C. johnstoni*; dehydration of the embryo being counteracted by increased absorption of water from the allantois. If so, the major ion concentrations would not be significantly affected by dehydration, as was found.

The decrease in the amount of soluble urate in allantoic fluid occurred earlier in eggs with air spaces than in those without them (70-75 days MA_{30} versus 80-85 days MA_{30}), which strongly suggests precipitation of urate salts (no crystalline material suggestive of uric acid was ever noted in any egg). This is supported by Whitehead (1987) who noted an increase in the solid content of allantoic fluid from *C. johnstoni* eggs in the final 30% of incubation. Such precipitation would provide another pathway for sodium and potassium losses (as sodium and potassium urate), and perhaps some ions (e.g. chloride) can be lost through inclusion with urinary precipitates, as occurs in birds (McNabb and McNabb 1977). The decreased levels of bicarbonate in eggs with air spaces may also be indicative of precipitation, as high levels of ammonium (not measured here) would undoubtedly be in the allantoic fluid, and the formation of ammonium carbonate would be readily possible.

The processes mentioned would all help in maintaining osmolality (Fig. 9) in the face of increasing water losses due to dehydration. Indeed changes in osmolality (or perhaps correlated changes in pH) may trigger the earlier precipitation of urate salts in eggs with air spaces.

Chorio-amniotic Fluid

The amnion is fully formed by three days MA_{30} (Stage 3; Ferguson 1985) and completely encloses the embryo. Compared to the other extraembryonic membranes it is a small chamber, although its significance is by no means reflected in its size. In the avian embryo maximum weight of the amnion (0.3 g) occurs after 70% of incubation time, after which it remains relatively constant (Romanoff 1967); the volume of amniotic fluid follows a similar pattern (Romanoff 1967). We did not record amnion weights of *C. johnstoni*, but they probably change in much the same way as do those of birds.

The amount of amniotic fluid we collected from *C. johnstoni* at any one time never exceeded 3.0 cm^3, and was usually considerably less. This meant that small volumetric losses during extraction represented a significant proportion of the total, and we considered our samples to be inadequate for detailed analysis. In addition, it was difficult to be sure that chorionic and amniotic fluids had been effectively separated.

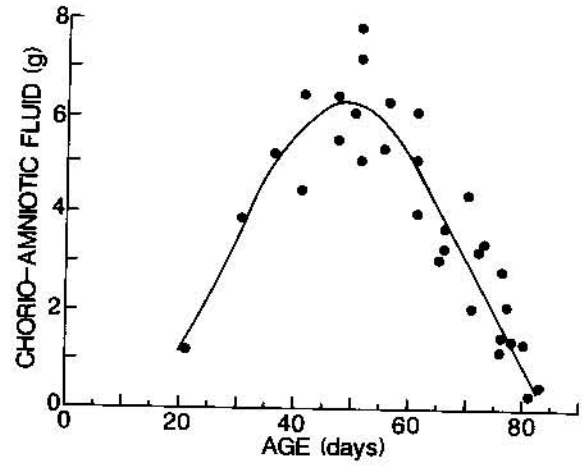
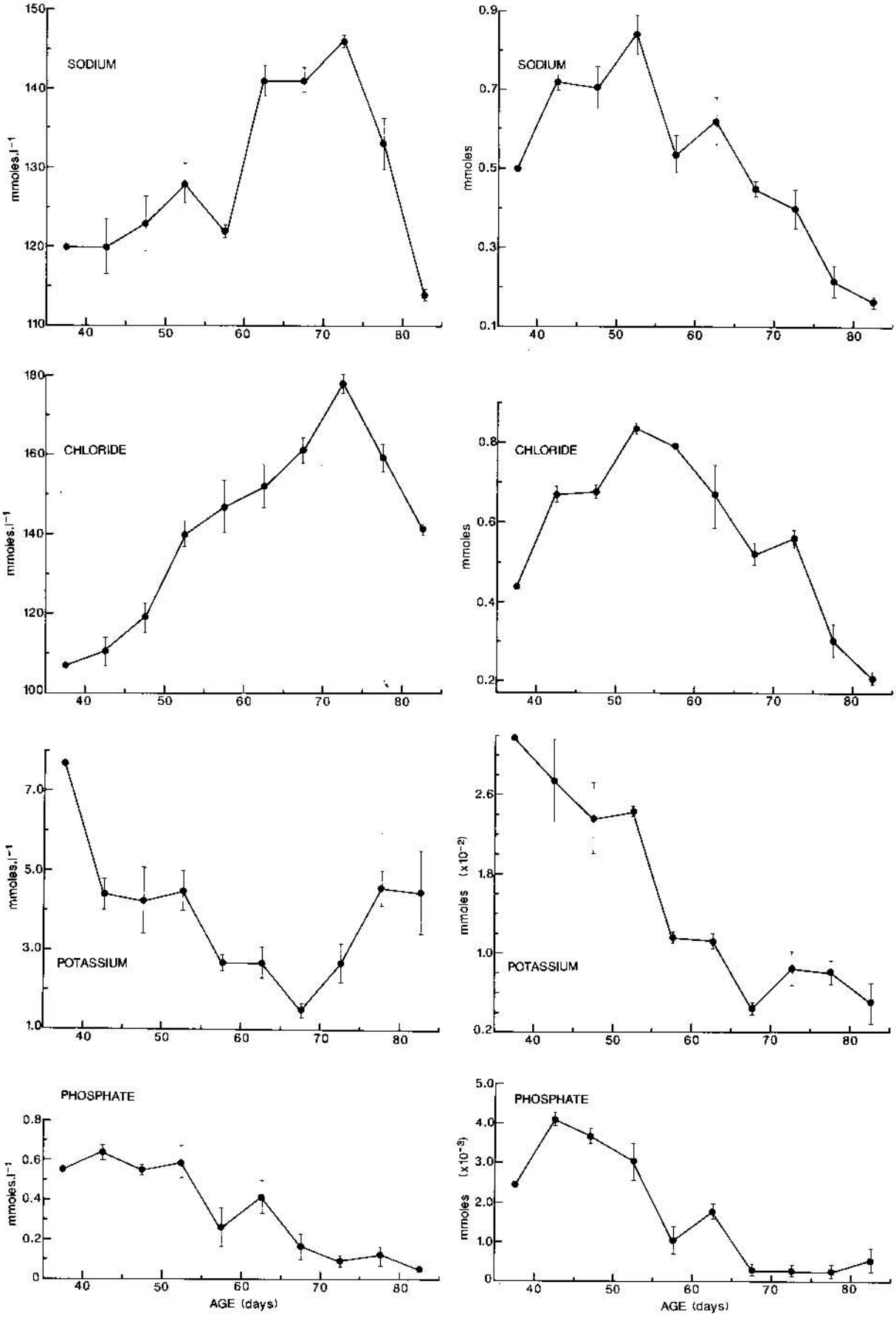


Fig. 10. Wet weight of chorio-amniotic fluid in *Crocodylus johnstoni* eggs incubated at 30°C. Data are standardized to mean egg weight (68.2 g).

The volume of the combined chorio-amniotic fluids increases up to about 50 days MA_{30} (Fig. 10; maximum volume in the mean sized egg = 6-7 cm^3). The time of maximum volume coincides with the time of maximum volume of allantoic fluid, as in birds (Romanoff 1967; Stewart and Terepka 1969). By 85 days (at 30°C) there was a negligible amount of chorio-amniotic fluid remaining.

Where good samples of amniotic and chorionic fluids were obtained from the one egg, analyses indicated that:

1. The concentration of calcium is on average 3.0 times greater in amniotic than in the chorionic fluid;



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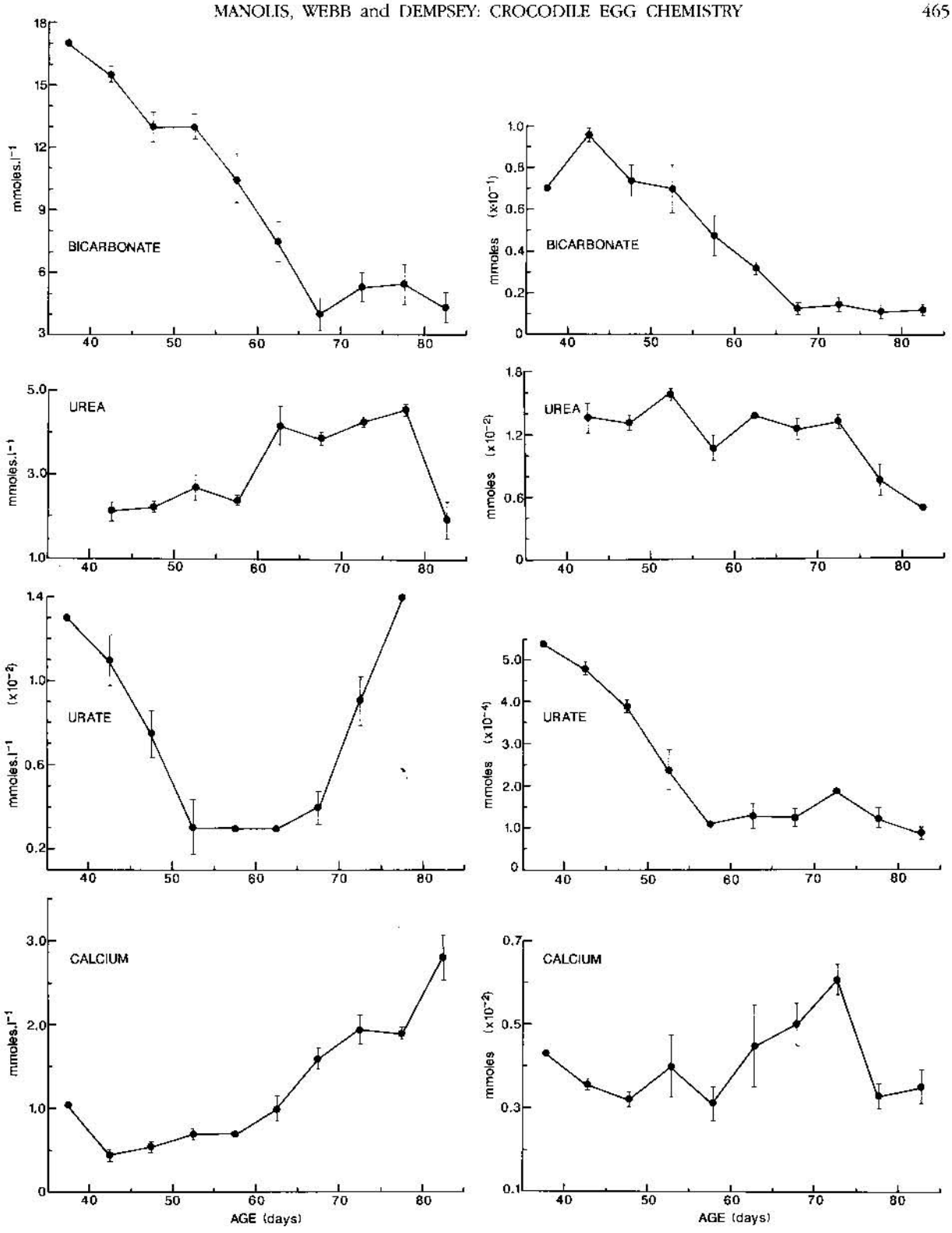

Fig. 11. The concentrations and relative amounts of the major ions in the chorio-amniotic fluid of *Crocodylus johnstoni* eggs. Data from incubation temperatures between 28°C and 34°C are standardized to 30°C relative ages (MA_{30}), and all data are standardized to mean egg weight (68.2 g). Means with one standard error refer to five day intervals of MA_{30}.

2. Bicarbonate concentration is on average 3.7 times greater in chorionic than in amniotic fluid;
3. Negligible amounts of soluble urate are in the amniotic fluid; and,
4. Concentrations of all other ions are similar in both fluids.

When analysing the ions of combined chorio-amniotic fluid (Fig. 11), the concentrations of major ions are probably indicative of the general levels in both compartments, whereas calcium, bicarbonate and urates reflect the situation more in one than the other. Absolute quantities of ions were calculated from the total volume of amniotic and chlorionic

fluid and thus the figures do not represent either of the two fluids alone.

As in allantoic fluid, the concentrations of sodium, chloride and calcium increase with time. Sodium and chloride ion concentrations peak at 50-55 days MA_{30}, and the amount of calcium increases markedly after 60 days MA_{30}. In domestic fowl eggs, sodium and chloride concentrations in the amnion follow a similar pattern (Romanoff 1967). Calcium concentrations on the other hand, show a decrease in the final stages of incubation (Romanoff 1967). All ions, in both *C. johnstoni* and domestic fowl eggs, show increasing quantities, a peak, and then a decline late in incubation.

Inorganic phosphate, bicarbonate and potassium show decreasing concentrations until about 65-70 days MA_{30} after which potassium concentrations increase and those of inorganic phosphate and bicarbonate tend to stabilise. In contrast to this situation, potassium and inorganic phosphate concentrations in the eggs of domestic fowls increase during the earlier stages of incubation (Romanoff 1967): the significance of these differences is unclear.

Inorganic phosphate and potassium are in minor concentrations in the chorionic and amniotic fluids, and thus sodium and chloride are probably the main ions associated with water movements, as in the allantois. Increasing chorio-amniotic fluid volume (to 50 days MA_{30}) is associated with increasing sodium and chloride levels, and decreasing volume with decreasing levels. Sodium and chloride ions could be regulated by ion pumps or by the embryo; that chorio-amniotic fluid is not in ionic equilibrium points to active processes being involved (Simkiss 1980).

It is more than likely that water within the amnion is utilized directly by the embryo rather than being transported to the allantois. [Water content of yolk is decreasing at this time (Fig. 4a) and the allantois is the only remaining compartment]. That the osmolality of chorio-amniotic fluid increases between 40-75 days MA_{30} (Fig. 9b) is primarily the result of changing amounts of sodium and chloride.

The amount of urea in amniotic fluid remains relatively stable throughout most of the incubation period (Fig. 11). Soluble urates are in minor concentrations in the chorionic fluid and in negligible amounts in the amniotic fluid. Creatinine levels were also in very low quantities generally (0.02-0.04 mmoles l^{-1}), and in most cases were so low that they could not be measured. In eggs of domestic fowls, where uric acid is the main excretory product, urea levels increase late in incubation and urate levels increase throughout incubation (Romanoff 1967). However, urea is a significant excretory product of *C. johnstoni*. Compared to the allantois, little of the total nitrogenous waste is contained in either the amniotic or chorionic fluids (Figs 7 and 10).

Calcium

Calcium is the main element required for ossification of the embryonic skeleton. Its utilization by embryos of the domestic fowl has long been studied (Johnston and Comar 1955; Romanoff 1967), but comparable data for oviparous reptiles have only recently been reported (M. J. Packard and G. C. Packard 1984; M. J. Packard *et al.* 1984a, b, 1985).

Some 60-80% of embryonic calcium requirements are obtained from the eggshell in three species of sea turtles (Simkiss 1962, 1967; Bustard *et al.* 1969), and 56% in the freshwater turtle *Chelydra serpentina* (M. J. Packard *et al.* 1984b): the remainder is supplied from the egg contents. However, in *Coluber constrictor*, 79% of embryonic calcium comes from the yolk (M. J. Packard *et al.* 1984a) and in the lizard *Amphibolurus barbatus*, 60% comes from the yolk (M. J. Packard *et al.* 1985). The only data available for crocodilians (*C. novaeguineae*) indicate that 63-70% of calcium is obtained from the eggshell, with the remainder coming from the egg contents (Jenkins 1975). We have no comparative data on these percentages for either *C. johnstoni* or *C. porosus*, but can shed some light on calcium mobilization within the allantois.

The patterns of calcium mobilization were the same at all temperatures, although the time scale varied, indicating that the process may be temperature dependent, as in the eggs of domestic fowls (Crooks and Simkiss 1975). The amount of calcium in the allantois between 10 and 50 days MA_{30} remains relatively stable, at a relatively low level (1.5×10^{-2} mmoles), but from 50 days MA_{30} onwards, it increases 10 fold (to 0.15 mmoles by 80-90 days MA_{30}; Fig. 8). This pattern mirrors the calcium content of developing reptile embryos (M. J. Packard and G. C. Packard 1984; M. J. Packard *et al.* 1984a, b, 1985), which increases during the period of rapid embryonic growth. Growth rate of *C. johnstoni* embryos increases markedly at around 50-60 days MA_{30} [6 g embryo; Webb *et al.* (1986b), Whitehead (1987)], and this is the same stage at which ossification of the skeleton begins in earnest (Ferguson 1982).

In the first 50% of incubation in domestic fowl eggs, 85-95% of embryonic calcium is derived from the yolk, and thereafter it comes mainly from the shell (Johnston and Comar 1955). Some 80% of the total calcium requirements are eventually derived from the shell (Simkiss 1967). As the dry weight of the shell of *C. johnstoni* decreases during incubation (Whitehead 1987), it would seem likely that significant amounts of calium are derived from it, especially in the last half of incubation.

In the embryos of domestic fowls, transport of calcium by the chorioallantois appears to be energy dependent, the ion being actively transported against a large chemical gradient (Moriarty and Terepka 1969; Stewart and Terepka 1969; Terepka *et al.* 1969). The process by which calcium is resorbed from the shell is oxygen dependent (Garrison and Terepka 1972) and the ectodermal cells of the chorion are believed to be the site at which it occurs (Moriarty and Terepka 1969; Stewart and Terepka 1969; Coleman *et al.* 1970; Garrison and Terepka 1972; Kyriakides and Simkiss 1975). The evidence to date therefore points to the chorion being the site of eggshell resorption. Active pumps would pass calcium through this membrane to the blood or directly into the allantois: Moriarty and Terepka (1969) suggested that another pump functions in the opposite direction, carrying calcium from the allantoic fluid to the blood supply.

Sodium, chloride, proton and calcium pumps in the chorioallantois of domestic fowl embryos are consistent with ion transport facilitating the removal of water from the allantois (Kyriakides and Simkiss 1975). However, in *C. johnstoni* the amount of calcium in the allantois does not decrease at any time, which suggests calcium is not an ion directly involved in water resorption. If calcium in the allantois of *C. johnstoni* is actively transported to the blood and thus to the embryo, then the rate at which calcium is transported from the eggshell to the allantois is greater than the rate at which it is removed.

The sudden increase in calcium levels in the allantois and the reduction in the dry weight of the eggshell (Whitehead 1987) are both indicative of significant amounts of eggshell calcium being mobilized during the development of *C. johnstoni* embryos. In *A. mississippiensis*, calcium is removed from the organic layer of the eggshell and is transported to the embryo via the chorioallantoic vasculature (Ferguson 1982); it would seem likely that the same pathways are used in *C. johnstoni* and perhaps all crocodilians.

Nitrogenous Excretion

With regard to nitrogenous excretory products, animals are often classified as: ammonotcles (mainly ammonia); ureoteles (mainly urea); or, uricoteles (mainly uric acid). In a relatively closed system such as an amniote egg, the excretory products become particularly important, because they can be potentially toxic to the embryo and require different amounts of energy to form them. Thus no energy is required to convert protein nitrogen into ammonia (Goldstein 1972), but ammonia is highly toxic. Ammonoteles are usually aquatic animals with ample water to flush or greatly dilute the ammonia (Maetz 1972). Where water is restricted as in terrestrial animals, ammonia is converted to urea or uric acid (Cragg *et al.* 1961). The formation of both requires energy and the loss of organic carbon, and thus it is only advantageous when water supplies are limited (Needham 1963).

All reptilian embryos studied to date have exhibited a preponderence of urea over uric acid (Tomita 1929; Clark 1953; Clark and Sisken 1956; Clark *et al.* 1957; Haggag 1964; G. C. Packard and M. J. Packard 1980, 1983; G. C. Packard *et al.* 1983, 1984), even though the adult form of excretion may be entirely different. *Crocodylus johnstoni* follows this same pattern.

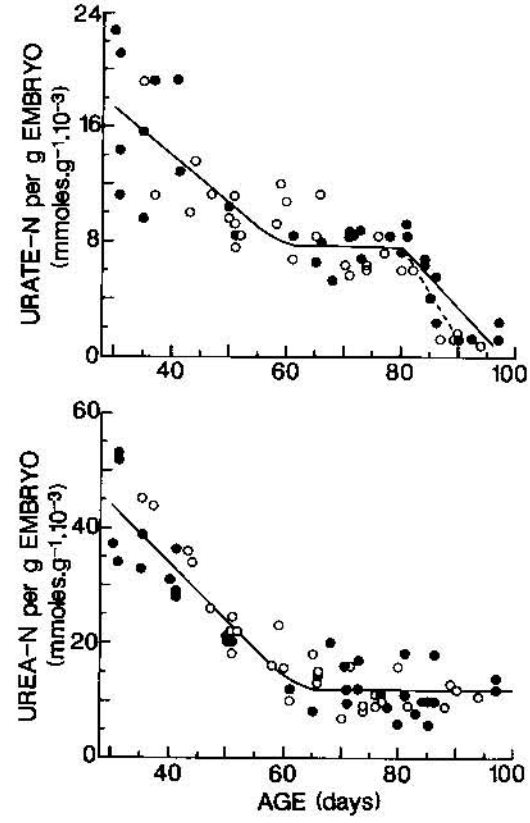

Fig. 12. Urea-nitrogen *(a)* and urate-nitrogen *(b)* per gram of embryo as a function of incubation time for *Crocodylus johnstoni* eggs. Data from incubation temperatures between 28°C and 34°C are standardized to 30°C relative ages (MA_{30}). Open circles indicate eggs with air spaces. Lines are eye-fitted before 60 days *(a,b)*, and after 80 days in *(b)*.

The rate of formation of urea with time in *C. johnstoni* does not decrease, although soluble urate essentially disappears at 80-85 days MA_{30}, and earlier (70-75 days MA_{30}) in eggs with air spaces. When excretory nitrogen is expressed as millimoles of nitrogen per gram of embryo (Fig. 12), the data indicate that after 60 days MA_{30} the amount of urea-N produced per gram of embryo remains constant at about 0.012 mmoles g^{-1}. Similarly, from 60-80 days MA_{30}, urate nitrogen is produced at a rate of 0.008 mmoles g^{-1}, although it then declines.

This decrease does not necessarily indicate lower rates of urate production, but rather the presence of less urate in solution in the allantoic fluid (Fig. 8). The amount of precipitated urate was indeterminable by our methods, but Whitehead (1987) found that the solid content of allantoic fluid did increase around this time. If the constant rate of urate nitrogen production between 60 and 80 days MA_{30} is indicative of urate production after 80 days MA_{30} (both dissolved and precipitated) then more excreted nitrogen is in the form of urea (60%) than urates (40%).

The high rates of production of both urea and urate in the early part of incubation are probably due to the rapid growth of extraembryonic membranes and a greater reliance on protein metabolism at this time. After 60 days MA_{30} the membranes are fully formed, and the excretory products are more specifically the by-products of embryonic growth and maintenance.

Creatinine comprises only a minor amount of the nitrogen excreted (Fig. 8). It is basically an energy storage compound, in the form of creatine, but it can also be excreted (Prosser and Brown 1961). *Crocodylus johnstoni* are therefore similar to other reptilian embryos studied to date in being ureotelic rather than uricotelic, but differ from avian embryos where urea is only a minor form of nitrogenous waste, and where uric acid is the major form (Needham 1963; Romanoff 1967).

Clark *et al.* (1957) studied excretion in *A. mississippiensis* embryos and found that 46.2%, 46.5% and 7.3% of excreted nitrogen was in the form of urea, ammonium salts and urates respectively. Their data show an unexplained decrease in urate nitrogen during incubation, which may be indicative of urate precipitation. If so, the contribution of uric acid may be underestimated and that of ammonia and urea overestimated. By using whole egg contents, a further problem may have been introduced, as ammonia cannot be regarded entirely as a waste product; it is also a metabolite from which urea and uric acid are formed and is involved in protein synthesis of the embryo and extraembryonic membranes (Haggag 1964).

Ammonia could very well be a major pathway for the excretion of nitrogen in *C. johnstoni*. We did not measure ammonia concentrations and thus cannot say one way or the other. Clearly however, any such analysis needs to address adequately the problem of isolating excretory ammmonia from that fulfilling other purposes. There seems no doubt that the allantois is clearly the major site for the storage of nitrogenous wastes, as only very small amounts of urea and urate were within the chorio-amniotic and subembryonic fluids.

Based on data from post-hatching crocodiles, Schmidt-Nielson and Skadhauge (1967) suggested that crocodilians have retained a uricotelic adaptation to a terrestrial existence and were forced to use other forms of excretion (ammonium salts) as an adaptation to an aquatic habitat. They also concluded that crocodilians probably do not have all of the enzymes needed for the urea cycle. The results of this study and that of Clark *et al.* (1957) indicate these hypotheses can be rejected; crocodilian embryos can dispose of large amounts of nitrogen in the form of urea. After hatching, crocodilians typically excrete small amounts of nitrogen in the form of urea, with the main products being NH_4HCO_3 and urate salts (Khalil and Haggag 1958; Cragg *et al.* 1961; Coulson and Hernandez 1964; Schmidt-Nielson and Skadhauge 1967; Grigg 1981). However, increased production of soluble ammonia occurs if large amounts of nitrogen need to be disposed of (Herbert 1981).

The predominant excretory product of post-hatching turtles is determined by the environment in which they live (Moyle 1949; Dantzler and Schmidt-Nielson 1966), whereas all turtle embryos so far studied tend very much towards ureotelism (Tomita 1929; G. C. Packard and M. J. Packard 1983; G. C. Packard *et al.* 1983, 1984). Needham's (1963) suggestion that the major form in which nitrogen is excreted by an animal is dependent upon the conditions under which its embryo had to live, is thus not substantiated with data from turtles or crocodilians. Embryos, like adults, appear to use the mode of excretion best suited to the particular environment in which they exist. What then does the mode of excretion of reptilian embryos tell us about the water relations of developing embryos?

The flexible-shelled eggs of snakes, most lizards and some turtles typically take up water from the nest environment, and are thus not limited entirely by the amount of water contained within them at the time of laying (Packard 1966). Hard-shelled eggs may also absorb water, but it in no way appears to be essential for normal development, and with *C. johnstoni*, occurs rarely in the wild: the embryo's water needs are supplied at the time of laying. If water is a limiting factor in hard-shelled eggs (Packard 1966), then one would expect water conserving mechanisms to have been selected for. The conversion of ammonia to the highly insoluble uric acid [0.384 mM at 37°C; Dantzler (1976)] would seem an essential step, as uric acid does not occupy osmotic space. Urea, although relatively non-toxic, still requires an ample supply of water for its storage. The preponderance of urea over urates in *C. johnstoni* eggs, implies that water economy is simply not a major problem. With *C. porosus* eggs, the major problem in the field is preventing too much water coming in. In both species, a considerable amount of water is retained in the allantois at hatching. In birds,

where no fluid remains in the allantois at hatching (Simkiss 1980), uric acid is the main waste product (Romanoff 1967).

Urates are an important part of the nitrogen metabolism cycle of reptiles (Minnich 1982) and uricotelism is the norm in lizards and snakes (Khalil 1948a, b, 1951; Haggag and Hassan 1968). Most of the urate is in the form of uric acid, with some as salts of ammonia (mainly), potassium and sodium urate (Minnich 1972). Even in the domestic fowl, where uric acid predominates, renally excreted ions are incorporated into urinary precipitates, possibly by physical means (McNabb and McNabb 1977). In crocodilians ammonium urate may be important, particularly when large amounts of ammonia are excreted (Dantzler 1978). The form of urates in *C. johnstoni* eggs is unknown.

We did not observe any crystalline structures (uric acid) in any eggs, although 'white solids' were sometimes present in the allantois. The major ions capable of forming the most common urate salts (sodium, potassium and presumably ammonium) are present in the allantoic fluid, and the sudden decrease in soluble urates in allantoic fluid could well be due to their formation and precipitation. If so, their earlier production in eggs with air spaces suggests it could be related to water economy. Potassium and sodium urate are very insoluble in water, although not to the degree of uric acid (Minnich 1972; Dantzler 1976, 1978).

How embryonic kidney function is affected by dehydration is unclear. Crocodilian embryos hatch from eggs with up to 30% weight loss (Bustard 1971; Lutz and Dunbar-Cooper 1984; Whitehead 1987), although post-hatching viability could be compromised at such extremes. Embryos from grossly dehydrated eggs have white deposits accumulated in the kidney tubules. Under mildly desiccating conditions (<15% egg weight) embryos appear to be kept preferentially hydrated (Hoyt 1979), and kidney function is probably not compromised

In adult vertebrates, the kidney regulates the composition of body fluids by controlling the excretion of ions, water and waste (Dantzler 1976). If the embryos' immediate environment (the amniotic fluid) remains unchanged, there may well be no stress placed upon the kidneys to retain homeostasis. That *C. johnstoni* are able to withstand a wide range of water losses is important if the embryo is to survive under changing conditions in the wild. The conditions in the nest at the time of laying are not the same as those during incubation or at the time of hatching.

Incubation temperature, and thus embryonic development rate, did not have any profound effect on the pattern of nitrogenous excretion in *C. johnstoni* embryos. It varied more with stage of development (morphological age).

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