

An Interim Method for Estimating the Age of *Crocodylus porosus* Embryos

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Abstract

On the basis of a reference series incubated at 30°C, methods for assigning unknown-aged embryos an approximate '30°C age' are derived. Determining real age is confounded by a paucity of information on the nest environment and its influence on embryonic development rate. Preliminary constants relating development rate to temperature (derived from studies with *C. johnstoni*) were tested with *C. porosus* and, as an interim measure, have practical utility in both the laboratory and the field.

Introduction

In northern Australia, *Crocodylus porosus* nest during an extended period (November to May-June; Webb *et al.* 1983b), encompassing the wet season (November to April-May). Because most nests are in remote, partly inundated areas, which are difficult to patrol regularly (Webb *et al.* 1977, 1983b; Magnusson 1980; Magnusson *et al.* 1978, 1980), aging wild embryos is one of the only practical methods available for quantifying the time relations of the nesting process.

To this end, Magnusson and Taylor (1980) presented embryo diagrams and descriptions against which unknown-aged embryos could be staged, but few data allowing 'stages' to be converted to 'ages' exist for any crocodylian (Ferguson 1983). There is a paucity of information on embryonic development rates and the extent to which they are influenced by the temperature and the gaseous and moisture environments of incubation (Ackerman 1980; Magnusson and Taylor 1980; Lutz and Dunbar-Cooper 1982; Ferguson 1983). In addition, 'staging' older embryos, on the basis of morphological characters alone, is not necessarily the best guide to aging, because the main changes in these larger embryos are increases in size (Voeltzkow 1902; Reese 1915; Magnusson and Taylor 1980; Ferguson 1983), and the size at hatching is a function of egg size (Webb *et al.* 1983a, 1983b).

The present report describes an interim method for aging embryos which extends Magnusson and Taylor's (1980) work by establishing a single standard series of embryos incubated at near-constant temperature (30°C), and by utilizing development rate-temperature coefficients to make gross corrections for incubation temperature. That major corrections are necessary is suggested by the development rate of *C. johnstoni* being halved by a drop in temperature from 30 to 26°C. Incubation times for *C. porosus* in the field are known to range from at least 71 days (Webb *et al.* 1983b) to 114 days (Magnusson 1979b).

The development of the opaque band (on the external surface of the egg), is also briefly described here. With *A. mississippiensis* (Ferguson 1982, 1983), *C. johnstoni* (Webb *et al.* 1983a) and perhaps all crocodylians, it can be used as an external indicator of embryo age, and often as a means of identifying infertile eggs (no band develops on them).

We used the method as described during a study of *C. porosus* nesting in freshwater swamps (Webb *et al.* 1983b), and feel that by extending Magnusson and Taylor's (1980) guide, it may be of use to others. *C. porosus* is a widely distributed crocodylian (India and south-east Asia in the north, to Australia in the south) which in some areas is considered endangered (Groombridge 1982).

The limitations of the method as described here should also be clearly recognized. In the absence of quantitative data we have accepted a number of untested assumptions, each of which merits detailed examination. *C. porosus* eggs were not readily available on the day of laying, and the detailed embryological studies ideally required could not be carried out within a reasonable time. Our primary aims in the present study were to extend the utility of Magnusson and Taylor's (1980) guide, and to identify areas where future work could lead to more precise aging.

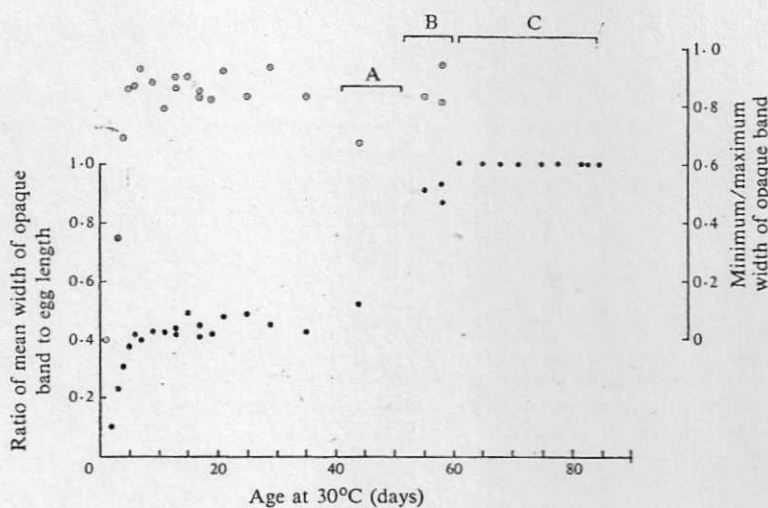


Fig. 1. The relationship between mean opaque band width as a ratio of egg length (●), and embryo age in the 30°C reference series. Minimum–maximum opaque band width (○) indicates how the band develops with regard to uniformity of width. A, period of rapid growth of opaque band, more on one side than on the other. B, opaque band almost complete at one end. C, opaque band complete to both ends.

Methods

30°C Reference Series

A clutch of 42 eggs, laid in captivity between 1600 and 0800 h on the night of 19–20 November 1980, was collected on 20 November 1980 and transported to an incubator. The upper surface of each egg was marked at the time of removal from the nest, and the eggs were repacked in a shallow layer of moist nest material at approximately the same orientation at which they had been in the nest. Eggs were considered to be much better aerated than in most nests. The incubator was set at 30°C ($\pm 0.4^\circ\text{C}$); temperature measured at the top of the incubator) and humidity was maintained at a high level with trays of water but was not measured. Mean egg dimensions were: length (EL), 81.3 ± 2.1 mm (SD); width 47.8 ± 0.6 mm; weight, 113.8 ± 3.1 g (mean EL from 53 clutches was 79.7 ± 4.9 mm; Webb *et al.* 1983b). During the later stages of incubation it is possible that metabolic heat may have increased clutch temperature ($<1^\circ\text{C}$) without it being indicated on the thermometer.

One egg was preserved at 1400 h on 20 November 1983 and a further egg preserved each day for a week. Eggs were then preserved each second day until 10 December 1980, then each fourth day and finally each week until hatching (10 February 1981).

Altogether, 30 live embryos were obtained, nine additional ones hatched, two failed during development (one egg was broken at its apex), and one appeared to be either infertile or had died before 1 day of age. With larger embryos a window was opened in the shell to determine whether the embryo

was alive, and smaller embryos which were translucent and firm after preservation were assumed to have been alive (dead ones are opaque and flaccid). The preservation technique was that suggested by Ferguson (personal communication), in which 20% formalin was injected into both ends of the egg before it was submerged in 20% formalin.

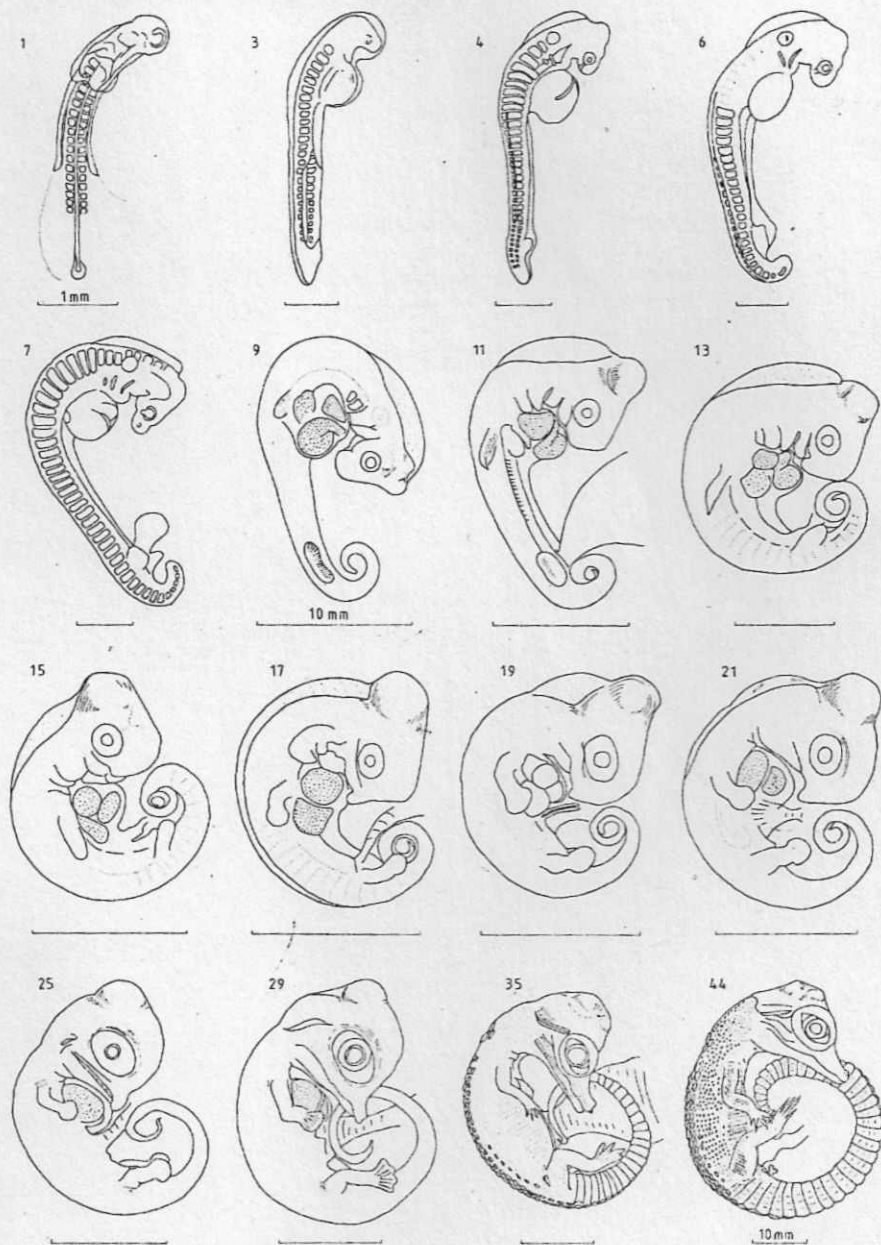


Fig. 2. Diagrams of known-age *C. porosus* embryos incubated at 30°C. Numbers are days.

Embryo Removal and Measurements

With the reference series, the presence or absence of the opaque band around the midpoint of the egg was noted, and if it were present its maximum and minimum widths were measured with calipers (Ferguson 1982).

The technique for removal of embryos we used varied from that described by Magnusson and Taylor (1980). The upper two-tenths of the eggshell (with the mark delimiting the upper surface of the egg usually in the centre) was removed by cutting through the shell with scissors, gently lifting the severed section, and severing the embryonic membranes attached to the margins of the opaque band. The smallest embryos in the reference series were already attached to the inner surface of the shell within a distinct attachment disc (younger stages are small discs of tissue on the upper surface of the yolk).

Embryo dimensions were measured in millimetres, with an optical micrometer (< 15 mm) or calipers (> 15 mm), and care was taken to ensure the caliper jaws did not indent soft tissue. The measurements taken on the reference series varied with embryo size and stage of development, and included:

Head length (HL): the maximum length of the head, measured (on all embryos) to the snout tip rather than the caruncle tip if the latter were present. Once the cranial platform was distinct (later embryos) its medial posterior edge was used as a posterior reference point.

Snout-vent length (SVL): the straight-line distance between the tip of the snout and the anterior end of the cloaca; measured on larger embryos, after scutellation was complete (> 55 days).

Eye length (EYL): the maximum length of the eye, measured from the inner corners of the eyelids once they had formed; measured on all embryos but of limited use once the eyelids are formed.

Yolk volume: the volume of yolk not absorbed into the body (measured by water displacement; in millilitres) in eggs which contained embryos more than 20 days of age.

Attachment disc dimensions: Length and width of the attachment disc on the upper surface of the egg; measured when embryos were very small (< 6 days) and the disc was distinct.

Unless otherwise stated, variation around means is expressed as \pm one standard deviation throughout the paper.

Table 1. Linear regression coefficients for predicting the age in days of *C. porosus* embryos incubated at 30°C

Measurements are expressed as ratios of egg length. *A*, intercept; *B*, slope; SEE, the standard error of prediction

No.	Measurement needed	<i>A</i>	<i>B</i>	\pm SEE	Applicable age
1	Head length	+6.56	122.13	1.3	36-62 days
2	Snout-vent length	+5.7	45.72	1.6	62-82 days
3	Head length	+3.7	135.35	0.9	7-36 days
4	Mean diameter of attachment disc	-0.5	16.83	0.5	0-6 days

Results

The Reference Series

(i) *The opaque band*

The eggs were uniformly translucent when laid, but developed an opaque spot on the upper surface within 24 h. This spot extended laterally and eventually formed a complete band (2 days), which expanded and remained distinct until approximately 45-55 days, when it extended first to one end of the egg, then to the other (Fig. 1).

(ii) *The embryos*

Morphological changes occurred rapidly during the first half of the incubation period (Fig. 2), after which major external changes were increases in embryo size and a steady reduction in the volume of external yolk (Fig. 3). As embryo size is related to egg size (Ferguson 1983), embryo measurements were expressed as ratios of egg length and plotted against known age. Plots with linear relationships over discrete periods were used to generate a series of formulae (by regression analysis) for predicting embryo ages from their morphometric ratios (Table 1). The relationship between eye length and age (Fig. 4) was plotted because in eggs containing small dead embryos the eye is often the only recognizable structure which can be measured, and thus the only indication of stage at death.

Assigning Unknown-aged Embryos a Standard Age

When incubated under the same temperature and moisture conditions, *A*.

mississippiensis embryos show minimal variation in the relationship between age, embryo development stage and the shape and proportions of the opaque band (Ferguson 1983); the relationships between them are highly predictable. Accordingly, the *C. porosus* reference series was used to derive approximate '30°C ages' of unknown-aged embryos.

Up to 44 days, embryos were mainly aged by morphological features (Fig. 2), although when only part of an embryo was available (damaged or decomposed embryos) the morphometric formulae (Table 1) and occasionally eye size (Fig. 4), were used. When the same embryos were aged by means of both morphological features and measurements (expressed as ratios of egg length), variation between the two estimates reached a maximum of 3 days at 44 days of age, and was generally within 2 days.

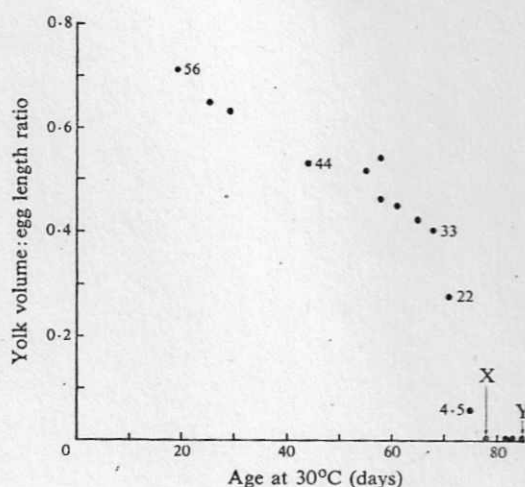


Fig. 3. The relationship between the volume of yolk outside the embryo (in millilitres), as a ratio of egg length (in millimetres), and age at 30°C. Numbers indicate selected yolk volumes, in millilitres. X, egg could hatch after this. Y, egg did hatch.

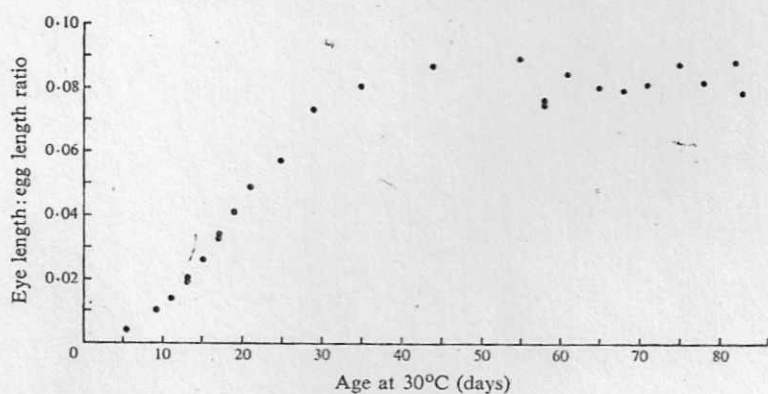


Fig. 4. The relationship between eye length as a ratio of egg length, and age at 30°C.

The morphometric formulae were used for embryos 45–85 days of age, and in most cases predicted ages were within a week of what was indicated by the extent of the external yolk volume (Fig. 3). Marked inconsistencies (≥ 7 days) between formulae predictions and remaining yolk volume occurred when embryos were structurally abnormal; such individuals could not be adequately standardized against the reference series.

Animals with the yolk sac just enclosed within the body were standardized at 78 days (some hatched and survived), and 85 days (the mean age at which animals in the reference series were hatched) when the ratio of umbilical scar width to egg length was 0.08

(approximately 6 mm wide in the average *C. porosus* hatchling). It should be noted that some hatchlings in the reference series were hatched by hand at 85 days; they might well have remained in the eggs for a longer period.

Table 2. Predicted and observed development rates in 14 wild nests of *C. porosus*

All ages are given in days

Clutch No.	When located				When collected			
	30°C age	Egg temp. (°C)	Correction factor	Estimated real age	Predicted 30°C age	Real 30°C age	Error (days)	Temp. change (°C)
M5	24	29.8	1.02	25	45	47	+2	+0.2
M13	58	28.8	1.14	66	75	82	+7	+3.3
M20	2	31.0	0.95	2	35	22	-13	-2.4
M24	44	31.3	0.94	41	49	48	-1	-
M22	16	30.8	0.95	15	36	40	+4	0.3
F3	1	32.6	0.88	1	79	78	-1	0.0
F5	37	33.4	0.84	31	85	83	-2	+0.5
F14	46	32.2	0.90	41	78	65	-13	+1.0
F21	14	33.2	0.85	12	91	87	-4	-3.0
F25	34	33.2	0.85	29	93	82	-11	-5.2
F26 ^A	2	29.0	1.10	2	47	11	-36	-5.0
F32	50	29.6	1.04	52	63	51	-12	-3.1
R3	14	32.6	0.88	12	80	69	-11	-4.6
R4	50	33.3	0.85	42	81	83	+2	-

^A Only one abnormal individual hatched from this nest, and it died on hatching; extremely cool temperatures appeared to have essentially stopped development.

The Relationship Between 30°C Age and Real Age

At the present time there appear to be no data available with which the development rate of any crocodylian species can be accurately predicted from the combined temperature, moisture and gaseous environment of incubation. However, for both *A. mississippiensis* (Ferguson 1983) and *C. johnstoni* (Webb et al. 1983a), temperature appears to have a

Table 3. Real and predicted development rates for *C. porosus* embryos at different constant temperatures

All eggs were incubated in sealed plastic bags with an open bottle of water; they may have had reduced oxygen availability. Ages are in days; Vegn, vegetation; TDC, temperature-development rate correction

* Measurement to hatching

Clutch No.	Medium	30°C age at start of incubation	N	Incubation temp.	TDC	Days of development	Predicted 30°C age	Real 30°C age
M.7*	Vegn	3	8	28.0 ± 0.1	1.30	109	84	79
Y.1	Sand	29	1	28.4 ± 0.4	1.20	39	62	60
Y.1	Sand	1	1	28.8 ± 0.6	1.15	35	30	29
M.22*	Vegn	40	21	30.0 ± 0.1	1.00	50	90	85
Y.1	Sand	30	1	30.2 ± 0.2	1.00	30	60	59
Y.1	Sand	32	1	31.7 ± 0.3	0.92	27	61	62
M.7	Vegn	3	1	34.0 ± 0.1	0.81	34	42	45

profound controlling influence. *C. johnstoni* eggs incubated at 34°C developed 0.81 times faster than at 30°C, whereas at 26°C they developed 2.1 times slower. At 26°C, 13 embryos between 8 and 88 days real age also had a linear relationship with their 30°C age ($r^2 = 0.99$; $P < 0.001$; Webb et al. 1983a).

In the absence of data for *C. porosus*, approximate temperature-development rate coefficients were derived by interpolating between these values (by an eye-fitted curve: 34°C, 0.81; 33°C, 0.86; 32°C, 0.91; 31°C, 0.95; 30°C, 1.00; 29°C, 1.1; 28°C, 1.3; 27°C, 1.6; 26°C, 2.1). This was not intended to imply a high level of precision, but rather to give a standardized method for compensating.

When these coefficients were applied to eggs incubating in the field (Table 2; on the basis of the mid-clutch temperature measured when a nest was first located) development rate was usually underestimated. This could be partly attributable to falling nest temperatures (F26 dropped to 24°C), although not in all cases (F14); clearly many other variables (including a need for specific *C. porosus* constants) are involved.

Since the study was completed, the same *C. johnstoni* constants have been applied to small groups of *C. porosus* eggs incubated under different conditions in a variety of pilot studies (Table 3). Predicted and observed 30°C ages reached a maximum deviation of 5 days (when development was extended to hatching), which was considerably less than if no corrections had been applied. These data also indicate the direction in which the specific *C. porosus* constants will vary from the *C. johnstoni* constants used, namely slightly faster development at lower temperatures and enhanced development at higher temperatures.

Discussion

In the field, incubation times for *C. porosus* are known to vary between 71 days (Webb *et al.* 1983b) and 114 days (Magnusson 1979b), and there would seem little doubt that this is related to the highly variable nest temperatures (Table 2; Magnusson 1979a; Webb *et al.* 1983b). Adequately accounting for temperature is an obvious consideration when attempting to relate 'time' to developmental stage.

The approach taken in the present study was to make gross corrections for temperature which, from a practical point of view, allowed us to plan when to make a circuit revisiting nests. For example, when nest F21 (Table 2) was first visited, an approximate development schedule was mapped on the basis of the nest temperature recorded at that visit. Knowing that incubation was extending into a cool period of the year, we expected that the development time would be prolonged, rather than shortened, and thus we could delay the circuit on which it was to be revisited.

Similarly, the magnitude of the errors recorded when eggs were incubated in the laboratory (Table 3) was a considerable advance over those without compensation. The clutch from M.7 (Table 3) had a real age of 112 days when its 30°C age equivalent was 79 days (prediction error was 5 days).

A first estimate of specific *C. porosus* constants can be derived from the data in Table 3; however, at this preliminary stage we considered there to be little utility in altering one variable in isolation, when a suite of interrelated variables need attention. The 30°C reference series needs to be established with a number of clutches from different females, and with more precise incubation temperatures. Morphometric relationships need to be established on longer series of embryos, and compensation for egg size investigated in depth. *C. porosus* developmental constants need to be derived under strict incubation conditions, with temperature, moisture and the gaseous environment controlled. The relationship between development rate and age at different temperatures (assumed to be linear here) needs to be quantified. Embryological stages need to be described in equivalent detail to those of *A. mississippiensis* (Ferguson 1983). From the point of view of predicting the real age of embryos from wild nests, there is a need for detailed investigations of the nest environment and factors influencing variability in it.

Using the present investigation as something of a pilot study and practical guide, we have now initiated (in collaboration with Mark Ferguson) a series of detailed, long-term investigations. We are hopeful that these will lead to a far more precise understanding of the developmental biology of both *C. porosus* and *C. johnstoni*, and eventually to a more precise

method of aging embryos and predicting development rates. In the interim, the preliminary results are presented here in the hope they will be of use to others working with *C. porosus* nests and embryos.

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References

- Ackerman, R. A. (1980). Physiological and ecological aspects of gas exchange by sea turtle eggs. *Am. Zool.* 20, 575-83.
- Ferguson, M. W. J. (1982). The structure and composition of the eggshell and embryonic membranes of *Alligator mississippiensis*. *Trans. Zool. Soc. Lond.* 36, 99-152.
- Ferguson, M. W. J. (1983). The reproductive biology and embryology of crocodylians. In 'Biology of the Reptilia'. Vol 14. (Eds C. Gans and F. S. Billet.) (Academic Press: London.) (In press.)
- Groombridge, B. (1982). I.U.C.N Amphibia-Reptilia Red Data Book. Part 1. Testudines, Crocodylia, Rhynchocephalia. (I.U.C.N. Publications: Gland, Switzerland.)
- Lutz, P. L., and Dunbar-Cooper, A. (1982). The nest environment of the American crocodile. Rep. Natl Park Serv., Everglades Natl Park, Florida.
- Magnusson, W. E. (1979a). Maintenance of temperature of crocodile nests (Reptilia, Crocodylidae). *J. Herpetol.* 13, 439-43.
- Magnusson, W. E. (1979b). Incubation period of *Crocodylus porosus*. *J. Herpetol.* 13, 362-3.
- Magnusson, W. E. (1980). Habitat required for nesting by *Crocodylus porosus* (Reptilia: Crocodylidae) in northern Australia. *Aust. Wildl. Res.* 7, 149-56.
- Magnusson, W. E., Grigg, G. C., and Taylor, J. A. (1978). An aerial survey of potential nesting areas of the saltwater crocodile, *Crocodylus porosus* Schneider, on the north coast of Arnhem Land, northern Australia. *Aust. Wildl. Res.* 5, 401-15.
- Magnusson, W. E., Grigg, G. C., and Taylor, J. A. (1980). An aerial survey of potential nesting areas of *Crocodylus porosus* on the west coast of Cape York Peninsula. *Aust. Wildl. Res.* 7, 465-78.
- Magnusson, W. E., and Taylor, J. A. (1980). A description of developmental stages in *Crocodylus porosus*, for use in aging eggs in the field. *Aust. Wildl. Res.* 7, 479-85.
- Reese, A. M. (1915). 'The Alligator and its Allies.' (Putnam and Sons: London and New York.)
- Voeltzkow, A. (1902). Beiträge zur Entwicklungsgeschichte der Reptilien. I. Biologie und Entwicklung der äusseren Körperform von *Crocodylus madagascariensis* Grand. *Abh. Senckenb. Naturforsch. Ges.* 26, 1-150.
- Webb, G. J. W., Buckworth, R., and Manolis, S. C. (1983a). *Crocodylus johnstoni* in the McKinlay River area, N.T. V. Nesting biology. *Aust. Wildl. Res.* 10, 407-20.
- Webb, G. J. W., Messel, H., and Magnusson, W. E. (1977). The nesting of *Crocodylus porosus* in Arnhem Land, northern Australia. *Copeia* 1977, 238-50.
- Webb, G. J. W., Sack, G. C., Buckworth, R., and Manolis, S. C. (1983b). An examination of *Crocodylus porosus* nests in two northern Australian freshwater swamps, with an analysis of embryo mortality. *Aust. Wildl. Res.* 10, 571-607.