

Early Developmental Arrest during Immersion of Eggs of a Tropical Freshwater Turtle, *Chelodina rugosa* (Testudinata : Chelidae), from Northern Australia

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Abstract

Freshly laid eggs of *Chelodina rugosa* survived for up to 12 weeks when immersed in water and subsequently underwent successful incubation and normal hatching. Embryonic development was arrested during immersion, remained arrested in an atmosphere of nitrogen, and recommenced when eggs were exposed to air. The hypoxic conditions during immersion appear to extend the arrest typical of turtle embryos during their period in the oviducts.

Freshly laid eggs of the temperate-zone *C. longicollis* died when immersed for longer than one week and eggs of both species died when immersed after post-laying embryonic development had commenced.

These results, supported by anecdotal and experimental evidence, suggest that *C. rugosa* lays its eggs in saturated or flooded ground in the late wet or early dry monsoonal season. Embryonic development presumably remains arrested until water levels drop and oxygen tensions in the nest rise by diffusion through the drying soil. Partly developed embryos in nests that are flooded after laying would perish. In contrast, *C. longicollis* of temperate Australia nests only in relatively dry substrates, and its eggs appear not to have evolved the capacity to withstand immersion.

Introduction

Recent studies have demonstrated a remarkable range of interactions between environmental conditions and developmental attributes in reptilian eggs. Rate of embryonic development, length of incubation period, hatchling size and coloration, sex and posthatching behaviour may all be directly influenced by the nest environment (Yntema 1978; Bull 1980; Ewert 1985; Packard and Packard 1988; Burger 1989; Deeming and Ferguson 1989a, 1989b; Janzen *et al.* 1990; Whitehead *et al.* 1990; Ewert and Nelson 1991). One of the least studied aspects of reptilian embryonic development is developmental arrest, and turtles possess a rich variety of ways to prolong the egg stage. Ewert (1985) recognised four types of developmental arrest. The first, termed pre-egg-laying arrest, occurs in the oviducts where embryos typically develop to the late gastrula stage and development is arrested until the eggs are laid. Cold torpor arrest is a suspension or near suspension of development because temperatures of the egg and embryos are too low for development to proceed. This form of arrest may be typical of autumn-nesting species of the temperate zones such as *Pseudemys floridana* (Jackson 1988) and *Chelodina expansa* (Goode and Russell 1968). Low temperature, while sufficient to retard development in many species, is not necessary in all cases. Embryos of some species undergo diapause where they advance through early development very slowly even though the incubation temperature is conducive to rapid development at later stages as well as to rapid development of all stages in related species

(Ewert 1985). In some species, such as *Deirochelys reticularia* from northern Florida, a specific stimulus such as chilling of the eggs is required to terminate the diapause (Ewert 1985; Jackson 1988). A fourth form of retarded development is delayed hatching, which grades into embryonic aestivation and involves late dormancy of fully formed embryos under warm ambient conditions (e.g. *Carettochelys insculpta*: Webb *et al.* 1986).

Essentially, pre-egg-laying arrest allows the embryo to wait until conditions are right for the female to nest. The other forms of arrest allow the embryo to wait out periods that may be unfavourable for normal development and to time its emergence with environmental conditions that are favourable for hatching and survival of hatchlings. Delayed hatching and embryonic aestivation terminated in response to a specific environmental cue, such as inundation (Webb *et al.* 1986), may compensate for differential developmental rates among eggs in a single nest and therefore permit synchronous hatching (Thompson 1989; Whitehead and Seymour 1990).

In this paper, we describe a new form of developmental arrest in an Australian freshwater turtle, *Chelodina rugosa*. This species is a long-necked chelid widely distributed in the wet-dry tropics of northern Australia (Cogger 1988), where it occurs in highest densities in shallow (<2 m) vegetated ephemeral water bodies. Rainfall throughout its range is markedly seasonal (Taylor and Tulloch 1985) and there is typically a dramatic rise in water levels during the monsoonal wet season and a fall in water levels during the following dry season. Many water bodies dry completely, depending on the time of onset, duration and intensity of the preceding wet season, all of which are highly unpredictable. *C. rugosa* survive the dry season by burying in the muddy bottom of their receding habitat and aestivating underground (Grigg *et al.* 1986). They nest between March and October before entering aestivation, laying between 7 and 19 hard-shelled ellipsoid eggs (Kennett, unpublished data).

Anecdotal information, provided chiefly by local Aboriginal people, suggests that eggs are laid in saturated soils or beneath free water. If true, levels of oxygen available to eggs following egg-laying are unlikely to be sufficient for embryonic development, and prolonged immersion of eggs in water or mud must presumably be accompanied by some form of embryonic arrest. We conducted a series of immersion experiments in the laboratory to test this hypothesis. As a comparison, we conducted similar experiments on the eggs of *Chelodina longicollis*, a related species of temperate-zone Australian turtle that nests only in relatively dry substrates (Vestjens 1969; Kennett and Georges 1990). We discuss the potential role of subaquatic nesting as a strategy for maximising reproductive output and enhanced clutch survival in an unpredictable environment.

Materials and Methods

Gravid specimens of *C. rugosa* were collected from ephemeral swamps and lagoons near Darwin between March and July of 1989 ($n=12$) and July 1990 ($n=2$). They were air-freighted alive to the University of Canberra, Australian Capital Territory. Three gravid specimens of *C. longicollis* were collected near Canberra in November 1989. Eggs of *C. rugosa* were obtained by dissection or hormonal induction after a period of 7–10 days in captivity to ensure adequate deposition of eggshell. Eggs of *C. longicollis* were obtained by hormonal induction immediately after capture as they were collected while in the early stages of nesting. In both species the eggs were induced by intracoelomic injection of synthetic oxytocin at a rate of 1 unit per 100 g body weight (Ewert and Legler 1978), with a further dose of 0.5 units per 100 g if no eggs were laid after 3 h. All eggs were weighed (to ± 0.1 g), their lengths and widths were measured (to ± 0.1 mm) and they were placed under treatment within 20 min of removal from the female.

As a control experiment, 49 eggs of *C. rugosa* and 6 eggs of *C. longicollis* were incubated in water-jacket incubators at a constant 30°C (range $\pm 0.2^\circ\text{C}$) on a bed of moistened vermiculite in plastic containers enclosed in sealable plastic bags to maintain a high but unmeasured humidity. One corner of each bag was snipped off to allow free gas exchange while holding water loss to a minimum.

Containers were weighed weekly and water added as necessary to maintain a constant ratio of three parts vermiculite to four parts water by weight. Addition of water was seldom necessary. Incubation of the control eggs commenced immediately on removal from the female. The appearance of an opaque white patch on the uppermost surface of the egg was taken to indicate that embryonic development had commenced (Thompson 1985) and was used to separate fertile eggs from eggs presumed to be infertile. Temperatures were monitored daily with a mercury thermometer calibrated against a NATA-certified thermometer (to $\pm 0.1^\circ\text{C}$), with the bulb placed amongst the eggs of one container.

In the first experiment, 78 eggs of *C. rugosa* and 34 eggs of *C. longicollis* were, for each species, systematically allocated across containers to form a single layer of eggs in each container. Distilled water was added to a depth of 4 cm above the eggs. All eggs obtained by dissection were immersed within 20 min of removal and all hormonally induced eggs immediately at the time of laying. The containers of *C. rugosa* eggs were placed in constant-temperature incubators set at either 26, 28 or 30°C and a small number of eggs, typically three from each treatment, were removed at 1-week intervals. The three temperatures were used to assess whether the influence of duration of immersion on egg survival was influenced by storage temperature, but eggs survived immersion beyond the maximum duration dictated by egg availability at each temperature, so no such comparison was possible. Data from eggs at the three immersion temperatures were pooled for analysis. All *C. longicollis* eggs were held at 30°C during immersion. The experiment was terminated when no further eggs remained. The period of immersion ranged from 2 to 12 weeks for *C. rugosa* and from 1 to 6 weeks for *C. longicollis*. After immersion, the eggs were incubated at 30°C, as described for the control eggs.

In the second experiment, 8 eggs of *C. rugosa* were immersed in distilled water for two weeks, then transferred immediately to an atmosphere of nitrogen where they remained for a further two weeks. The aim of this experiment was to determine whether it was water *per se* or the lack of oxygen that was the probable cause of developmental arrest. The nitrogen atmosphere was established by providing a glass vacuum chamber, sealed but for inflow and outflow stopcocks, with a steady flow of nitrogen gas for 6 h. Every two days, the system was refilled with nitrogen gas for 30 min. A high but unmeasured humidity was maintained in the chamber by placing the eggs on wire gauze above water. Apart from the periods of refilling, the eggs and apparatus were maintained at 30°C in a constant-temperature incubator.

In the third experiment, two groups of 5 *C. rugosa* eggs and two groups of 5 *C. longicollis* eggs were incubated at 30°C for 10 and 20 days, respectively, until after the appearance of the white patch which indicated that embryonic development had commenced. The eggs were then immersed in distilled water and held at 30°C for 10 days before being returned to normal incubation conditions as for the controls. The aim of this experiment was to determine whether immersion after the commencement of development differed in its effect from immersion immediately after egg-laying.

In all experiments, eggs and temperatures were checked daily and the date of first appearance of the white patch, rate of patch expansion and date of hatching emergence were recorded.

Several eggs of *C. rugosa* were opened during incubation: first, for an assessment of the level of development of immersed and control eggs at the time of patching; second, for an assessment of the condition of immersed eggs during incubation; and third, for a preliminary assessment of whether *C. rugosa* embryos enter a period of embryonic diapause (Beynon 1991). Unfortunately, this precluded a detailed analysis of trends in mortality with duration of immersion.

All statistical analyses were performed with the statistical package SAS (SAS Institute 1986) following the recommended procedures of Sokal and Rohlf (1981). All means are given with their standard errors, unless otherwise specified.

Results

The white patch appeared on eggs of *C. longicollis* that were immersed for 1 week on average 5.0 days after exposure to air, longer than the 2.3 days recorded for the control eggs (Table 1), though the difference was not significant (SNK test, $P > 0.05$). There was also no significant difference in the incubation periods under either treatment ($t = 1.32$, d.f. = 10, $P = 0.22$; Table 1). In the field, eggs of *C. longicollis* patch within 24 h (authors' observations), and the relative delay in patching for the control eggs may have resulted because they were obtained by hormonal induction.

Table 1. Results of immersion experiments with eggs of *Chelodina longicollis*

Days to hatching are taken from the day the white patch first appeared

Duration of immersion (weeks)	<i>n</i>	No. to patch	Days ^A to patch (range)	No. to hatch	Days ^A to hatching (range)
0 (control)	6	6	2.3 ± 0.3 (2-4)	6	69.6 ± 0.78 (67-73)
1	6	6	5.0 ± 1.1 (2-10)	6	67.9 ± 1.02 (64-71)
2	7	3	11.7 ± 1.3 (8-15)	0	—
3-6	21	0	—	0	—

^A Mean ± s.e.

All control eggs of *C. longicollis* and the eggs that were immersed for one week patched and completed incubation, whereas only 3 of 7 eggs that were immersed for two weeks patched and none survived to hatching. None at all patched or survived to hatching after longer periods of immersion. Time to patch for eggs that were immersed for two weeks (mean 11.7 days) was significantly greater than for eggs that were not immersed or immersed for one week only (ANOVA, $F=15.4$; d.f. = 2, 12; $P < 0.0005$, SNK test). After four weeks of immersion, eggs of *C. longicollis* began to swell, indicating uptake of water.

In contrast, eggs of *C. rugosa* survived up to 12 weeks immersion without swelling and, on subsequent exposure to air, developed to yield normal hatchlings. At 12 weeks, the supply of experimental eggs ran out, so tolerance of greater periods of immersion could not be tested but appears likely. There was no significant relationship between duration of immersion and time to patch ($F=0.24$, d.f. = 1, 88, $P=0.63$); however, eggs immersed in water then held in a nitrogen atmosphere patched significantly sooner (mean 5.6 days; Table 2) than those that were simply immersed (mean 6.9 days) before being allowed to

Table 2. Results of immersion experiments with eggs of *Chelodina rugosa*

Days to hatching are taken from the day the white patch first appeared. Egg mortality explains the discrepancy between the number of eggs that patched and the number opened or that hatched

Duration of immersion (weeks)	<i>n</i>	No. to patch (%)	Days ^A to patch (range)	No. opened	No. to hatch	Days ^A to hatch (range)
0 (control)	49	47 (95.9)	7.7 ± 0.3 (6-9)	18	24	97.8 ± 2.3 (76-114)
2, 3	14	13 (92.9)	6.2 ± 0.3 (5-9)	8	5	120.6 ± 6.1 (99-136)
4-6	25	21 (84.0)	7.1 ± 0.4 (4-10)	7	12	98.9 ± 12.3 (81-143)
7-9	25	21 (84.0)	6.5 ± 0.2 (4-8)	12	7	112.7 ± 12.3 (88-163)
10-12	14	14 (100.0)	7.4 ± 0.3 (5-10)	6	5	95.0 ± 9.0 (86-104)
Water/N ₂	8	7 (87.5)	5.6 ± 0.3 (5-7)	2	3	92.0 ± 11.0 (81-114)

^A Mean ± s.e.

continue incubation ($t=2.58$, d.f. = 95, $P<0.05$). This latter result was probably caused by partial drying of the eggshell while in the nitrogen atmosphere. There was no significant association between the number of weeks that eggs were immersed and the relative number of eggs that hatched ($\chi^2=3.7$, d.f. = 5, $P=0.59$). Incubation periods were highly variable (Table 2), suggesting that some form of delayed hatching or embryonic aestivation might be operating (Beynon 1991), and no significant difference in the mean incubation period across treatments could be demonstrated (ANOVA, $F=2.16$; d.f. = 5, 46; $P=0.075$).

Two control eggs, two eggs that were immersed for two weeks, two eggs that were immersed for eight weeks and two eggs that were immersed and kept in nitrogen were opened immediately after hatching for an assessment of embryo development. Although what appeared to be embryonic cellular material was present, in no case could a discrete embryo be located, and it was assumed that embryonic development had not proceeded beyond Yntema's (1968) stage 0 and certainly not beyond stage 3. Eggs of *C. rugosa* not subject to immersion but incubated from laying at 30°C develop to stage 10 after 10 days, stages 14–20 after 4 weeks and stages 11–22 after 8 weeks (Beynon 1991). The data for the immersed eggs therefore clearly indicate that embryological development is arrested during immersion, and the nitrogen experiment suggests that hypoxia maintains the arrest.

In the final experiment, all eggs died when immersed after development had recommenced (after 10 and 20 days incubation).

Discussion

Our results indicate that eggs of *C. rugosa* will survive immersion in water for at least 12 weeks. Embryological development is arrested during immersion and recommences when the eggs are exposed to air. Removal from water *per se* does not terminate the arrest, which was maintained when eggs were no longer immersed but held in a nitrogen atmosphere. We conclude from this that the arrest is almost certainly maintained by the hypoxic conditions brought about by immersion. Such hypoxic post-egg-laying arrest has not previously been reported for any species of turtle.

Turtle eggs immersed in water face two major problems: surviving the hypoxic conditions and avoiding excessive water uptake along an osmotic gradient. Turtle embryos may be preadapted to arrest under hypoxic conditions after egg-laying, because embryonic development is normally arrested at late gastrula stage in the oviducts (Pasteels 1937, 1957; Yntema 1968). Arrest in the oviducts is believed to be maintained by hypoxic conditions in the oviduct (Ewert 1985).

Excess uptake of water by turtle eggs incubated under wet conditions or in flooded nests can cause swelling, cracking of the calcareous outer layer and rupture of the shell membranes with consequential death of the embryo (Plummer 1976; Ewert 1985). There are several possible solutions to this problem. First, *C. rugosa* may have an unusual calcareous eggshell structure which mitigates against water uptake. Such is the case for the grebe *Podiceps nigricollis*, whose eggs have a shell with large internal pores tapering to small external openings, an arrangement that prevents excessive water uptake while allowing gas exchange during incubation in partially submerged eggs (Sotherland *et al.* 1984). No such arrangement of pores was evident in a study of the eggshell structure of *Chelodina expansa* (Woodall 1984), a close relative of *C. rugosa* (Legler 1981, 1985). Instead, *C. rugosa* may have impermeable, perhaps hydrophobic, shell membranes. Early embryonic development involves an intrinsic drying of the shell and membrane immediately above the embryo (Thompson 1985), which may alter the permeability or hydrophobic properties of the shell membranes. This would explain why, once development has commenced, immersion is fatal to the embryo. A third possibility is that material migrates out of the egg into the surrounding water thus reducing the magnitude of the osmotic gradient between the egg contents and the surrounding water. These possibilities warrant further investigation.

The ecological significance of the ability of *C. rugosa* eggs to survive extended periods of immersion after egg-laying also warrants further investigation. Its southern relative, *C. longicollis*, has a reproductive pattern typical of most temperate-zone freshwater turtles, with spring nesting and summer hatching (Vestjens 1969; Parmenter 1985; Kennett and Georges 1990). Freshwater turtles are able to accumulate reserves for reproduction well in advance of ovulation and egg-laying (Chessman 1978; Georges 1983). Nesting occurs when temperatures are suitable for adult activity, including nesting, while also ensuring that conditions prevailing during incubation are conducive to embryonic development and that hatchlings emerge when conditions are favourable to their survival. Nesting in the spring and early summer has presumably been an adequate compromise of these influences for *C. longicollis*.

The onset of spring each year is highly predictable within the range of *C. longicollis*, and the ovarian cycle can be readily attuned to produce eggs at the appropriate time. Furthermore, while *C. longicollis* is like *C. rugosa* in its preference for ephemeral waters (Chessman 1988) and can aestivate when waters recede (Chessman 1983), its typical response is to migrate overland to more permanent water when ephemeral ponds and swamps dry (Parmenter 1976; Kennett and Georges 1990). Hence, the turtles are typically active, and relatively dry soils are available and accessible, when conditions suitable for nesting prevail.

The situation for *C. rugosa* is more complex. Although it does not have to contend with the cold winter months of the temperate zones, rainfall throughout its range is markedly seasonal (Taylor and Tulloch 1985). There is typically a dramatic rise in water levels during the monsoonal wet season (December to March) and a corresponding drop in water levels in the wet-dry transitional months (April, May) and the following dry season (June to August). During the wet season, *C. rugosa* occupies an extensive network of floodplains covering thousands of square kilometres (Finlayson *et al.* 1988) and rapidly fluctuating water levels limit the availability of relatively dry ground suitable for nesting. Dry ground available early in the wet season may subsequently be flooded if late wet-season rainfall is heavy, and flooding is a major source of mortality for many egg-laying species that occupy the floodplains (Magnusson 1982; Webb *et al.* 1983; Whitehead and Tschirner 1990). In the dry season, many waterbodies dry completely and the turtles survive by burying in the muddy bottom of their receding waterbody and aestivating underground (Grigg *et al.* 1986). Were *C. rugosa* to adopt the nest-site preferences of *C. longicollis* and other freshwater species, then only a narrow window of time would be available for nesting—after the wet season waters had receded but before aestivation became necessary.

In itself, this is not an insurmountable problem, as the reproductive cycle could be timed so that nesting and subsequent embryonic development coincided with suitable conditions, brief though they might be. However, although the wet-dry rainfall cycle occurs reliably each year, there is considerable annual variability in the timing of the onset, the duration and the intensity of the wet and dry seasons. Much of the variation between years lies in the transition periods between seasons (Taylor and Tulloch 1985). The combination of a narrow window in time suitable for nesting and extreme unpredictability in when the window is open would make it very difficult for *C. rugosa* to persist in the floodplains if its nesting requirements were similar to those of other freshwater turtles.

Instead, we suggest that *C. rugosa* has evolved eggs that withstand immersion immediately after egg-laying to allow nesting in saturated mud or beneath water. In so doing, the inability to predict the timing of the wet-dry transition is allayed and a more protracted nesting period is possible. The evolution of this capacity will also enable females to 'store' eggs in the ground when conditions for nesting and incubation would otherwise be unfavourable, thereby freeing the females to exploit the period of high food availability by producing more clutches during an extended wet season.

When the ground eventually dries and oxygen reaches the eggs, conditions would become suitable for incubation and development would proceed. The hypoxic developmental arrest demonstrated in this paper would serve as a hedge against saturated or inundated ground

when floodwaters are receding, but not against rising water levels, because all eggs drowned when immersed after development commenced.

Until recently, support for the proposition that *C. rugosa* lays its eggs underwater came largely from the knowledge of Aboriginal people. Aboriginal people of the Roper River region report, for example, that *C. rugosa* nests in shallow water at the base of paperbark trees (*Melaleuca* sp.) (J. Roberts, personal communication). More recently, a field study involving radio-tracking gravid females has confirmed that *C. rugosa* nests underwater (Kennett *et al.* 1993).

While arrested at late gastrula stage in the oviducts, and presumably during the arrest after immersion in *C. rugosa* the embryo remains unattached to the egg membranes (Ewert 1985). After egg-laying, the embryo moves to the uppermost surface of the egg, development recommences, and the embryo and adjacent vitelline membrane becomes attached to the inner shell membrane (Mitsukuri 1894; Ewert 1979). Transporting turtle eggs often results in high mortalities (Limpus *et al.* 1979; Parmenter 1980) unless it is done in the first few hours after egg-laying (Mitsukuri 1894) or in the first 96 h if development is retarded by chilling (Harry and Limpus 1989). Movement after this time presumably results in rupture of the membranes supporting the embryo (Ewert 1979).

Refrigeration is not necessary for transporting the eggs of *C. rugosa* as Beynon (1991) successfully transported immersed eggs over 3000 km by commercial courier. As the arrest is maintained by immersion for at least 12 weeks, there would appear to be no biological limit to their shipment from collection sites to distant laboratories. If ways could be found to induce similar arrest in other species, perhaps by extending preovipositional arrest, then management programmes for endangered species requiring relocation of eggs could be greatly simplified.

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