Limited Oxygen Availability In Utero May Constrain the Evolution of Live Birth in Reptiles

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Submitted April 30, 2012; Accepted September 4, 2012; Electronically published January 4, 2013

Dryad data: http://dx.doi.org/10.5061/dryad.895jb.

ABSTRACT: Although viviparity (live birth) has evolved from oviparity (egg laying) at least 140 times in vertebrates, nearly 120 of these independent events occurred within a single reptile taxon. Surprisingly, only squamate reptiles (lizards and snakes) are capable of facilitating embryonic development to increasingly advanced stages inside the mother during extended periods of oviducal egg retention. Viviparity has never evolved in turtle lineages, presumably because embryos enter and remain in an arrested state until after eggs are laid, regardless of the duration of egg retention. Until now, the limiting factor that initiates and maintains developmental arrest has remained elusive. Here, we show that oviducal hypoxia arrests embryonic development. We demonstrate that hypoxia can maintain developmental arrest after oviposition and that subsequent exposure of arrested embryos to normoxia triggers resumption of their development. We discovered remarkably low oxygen partial pressure in the oviducts of gravid turtles and found that secretions produced by the oviduct retard oxygen diffusion. Our results suggest that an extremely hypoxic environment in the oviduct arrests embryonic development and may constrain the evolution of viviparity in turtles, with the reduced diffusive capacity of oviducal secretions possibly creating or contributing to this hypoxia. We anticipate that these findings will allow us to better understand the mechanisms underlying the evolutionary transition between reproductive modes.

Keywords: amniotic, arrested development, embryonic development, incubation, reptile, turtle.

Introduction

The evolution of the amniotic egg is regarded as the principle driving force underlying terrestrial exploitation by vertebrates, enabling the water-dwelling amphibian-like ancestor of amniotes to evolve into terrestrial lineages (Reisz 1997; Blackburn 2005). In some terrestrial vertebrate lineages, the amniotic egg has been conserved despite readaptation to aquatic habitats, while it has been abandoned by others in favor of the evolution of viviparity (Shine 2005). Viviparity is the internal embryonic development and birth of a relatively well-developed neonate, and the evolutionary transition to this reproductive mode has multiple independent origins in lizards and snakes but none in other reptile groups (Shine 2005). Viviparity is mainly a terrestrial reproductive strategy (Shine 1985; Blackburn 1985) and requires the development of specialized internal structures and physiological modifications that facilitate water and gas exchange between embryo and mother necessary to sustain development through to live birth (Guillette 1989; Crespi and Semeniuk 2004).

In contrast, oviparous reptiles lay eggs at varying, species-dependent stages of embryonic growth at or beyond the blastula stage (Bellairs 1991; Shine 2005). Extended egg retention beyond that normally observed in an oviparous species is presumably detrimental to embryonic development, because the embryos of many lizard species and all turtles will enter a preovipositional developmental arrest if this occurs (Ewert 1985; Miller 1985; Andrews and Mathies 2000). Developmental arrest is a remarkable but poorly understood life-history trait that involves the cessation of active embryonic cell division and growth in the oviduct, with development not resuming until after eggs are laid (Ewert 1985; Miller 1985; Andrews and Mathies 2000). Crocodilians are incapable of arresting embryonic development, and in these reptiles extended egg retention results in developmental deformity (Ferguson 1985; Ewert 1991).

The onset of preovipositional arrest, or the occurrence of developmental deformity in its absence, suggests that there is a factor that limits development in the oviduct and thereby constrains the evolution of viviparity in extant oviparous reptiles. Restricted gas and/or water availability in the absence of oviducal structures that permit their exchange is a plausible restraint likely to limit development (Guillette 1989, 1993). However, restricted oviducal water availability is not a limiting factor during *Sceloporus* lizard

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Am. Nat. 2013. Vol. 181, pp. 245–253.
0003-0147/2013/18102-53821$15.00.
DOI: 10.1086/668827

embryonic growth (Mathies and Andrews 1995; Andrews 2004). In addition, although reptilian eggs typically absorb water during growth, hydric conditions have a relatively minor impact on development inside rigid-shelled eggs when compared to that inside flexible-shelled eggs (Packard and Packard 1988; Packard 1991). Furthermore, the onset of preovipositional arrest in rigid- and flexible-shelled chelonian eggs occurs at the same development stage despite eggshell type (Ewert 1985), suggesting that restricted water availability is not the primary limiting factor for chelonians either.

Certainly, limited gas exchange may inhibit embryonic development in the oviduct. In relation to embryonic metabolic activity in the oviducal environment, hypoxia (O_2 depletion) is more likely to restrict oviducal embryonic development than is hypercapnia (CO_2 accumulation), because at an equivalent partial-pressure gradient, the diffusion capabilities of CO_2 exceed those of O_2 in aqueous surroundings (i.e., albumin and oviducal secretions; Withers 1992). Therefore, it is plausible that hypoxia limits embryonic development and so has constrained the evolution of viviparity in oviparous reptiles.

In order to establish whether restricted O_2 availability in the reptilian oviducal environment limits embryonic development, we investigated whether hypoxia affects active embryonic development, determined the oviducal oxygen partial pressure (Po_2) experienced by reptilian embryos, and assessed the rate of O_2 diffusion in reptilian oviducal secretions. Turtles are ideal study animals to investigate our proposed role for hypoxia as a developmental regulator because they are the only reptile group in which all species undergo a period of preovipositional developmental arrest (Ewert 1985; Miller 1985). We used three species of freshwater turtle, in addition to one species of sea turtle, to show that restricted oviducal O_2 availability limits embryonic development.

Material and Methods

Study Animals

Oviducal O_2 availability and the impact of hypoxia on embryonic development were assessed in three species of freshwater turtle—the western oblong turtle *Chelodina oblonga*, the eastern longneck turtle *Chelodina longicollis*, and the Murray River turtle *Emydura macquarii*—in addition to the green sea turtle *Chelonia mydas*. *Chelodina oblonga* were trapped from Lake Goolelall in Western Australia between October 1 and October 7, 2010, in baited, modified funnel traps. *Chelodina longicollis* and *E. macquarii* were trapped from Lake Coranderrck, Victoria (Australia), between October 10 and December 15, 2010, in baited fyke nets. Detection of gravid females was through manual palpation of the inguinal pocket. Those determined to be gravid with this method were later radiographed to confirm presence of eggs. Eggs and oviducal fluid samples were collected from *C. mydas* during oviposition on Heron Island, Australia, from December 12 to December 14, 2010, and oviducal oxygen tensions were recorded in gravid females from November 16 to November 25, 2011.

Effects of Hypoxia on Eggs in Preovipositional Arrest

In order to understand the role O_2 plays in the maintenance of preovipositional arrest, we devised a normoxic treatment and a series of hypoxic treatments in which eggs were incubated for various periods of time. We then compared the progression of development of the embryos in each of these treatments to assess whether the different incubation conditions affected the developmental rate of the embryos and their capacity to resume development following preovipositional arrest.

Twelve *C. oblonga*, nine *C. longicollis*, and six *E. macquarii* gravid females were induced to lay their eggs. Female *C. longicollis* were placed in individual 68-L (60 cm × 38 cm × 40 cm) containers, while female *E. macquarii* and *C. oblonga* were placed in individual 150-L (92 cm × 41.5 cm × 32.5 cm) containers so that each female was floating in enough water (28°C) to cover the shell. After turtles were allowed 1 h to acclimatize to their surroundings, they were given an intramuscular injection of synthetic oxytocin (Butocin, Bumac, Hornsby, Australia) at a dose of 15 IU/kg (Ewert and Legler 1978). Oviposition generally occurred within 20 min.

As eggs were laid, they were immediately patted dry with a paper towel and marked on the shell for identification with a graphite pencil. Eggs from all females of each species were randomly distributed across one normoxic and three hypoxic treatments. The normoxic treatment exposed eggs to ambient atmospheric O_2 levels from the time of oviposition throughout subsequent embryonic development. All eggs in the normoxic treatment were placed in a plastic container with a loosely fitting lid containing air holes, with approximately half the shell covered in moist sand with a water potential of approximately −150 kPa (determined by thermocouple psychrometry). Eggs in the hypoxic treatments were placed in a hypoxic environment immediately after oviposition and kept there for a period of 3, 6, or 9 days before being transferred to the same environment as the normoxic treatment for the remainder of development. Each treatment group was placed in a separate but identical GQF HovaBator incubator (model 1632; Grandview Management, Baldivis, Australia) at a constant temperature of 22°C.

The hypoxic treatments were created using airtight Perspex containers (Resi-Plex Plastics, North Geelong, Aus-

tralia) measuring 26 cm × 15.75 cm × 11 cm, with inflow and outflow valves at either end. Pure nitrogen gas (BOC Gases, Mt. Isa, Australia) was pumped at a constant rate into each chamber via the gas inlet, beginning approximately 3 min before introduction of the first egg, until valve closure. The gas was humidified by bubbling it through distilled water in a separate airtight flask. The lid of each chamber was lifted briefly and replaced after each egg was added. Eggs were positioned on wire mesh above 20 mL of distilled water in each chamber. Eggs were added to each chamber as they were laid, for up to 10 min after the first egg was introduced. At this time, the chamber was sealed and the nitrogen gas was allowed to flow through the chamber for an additional 3 min before both valves were closed, sealing the chamber. The O_2 tension of the gas escaping the chamber via the outlet valve was measured continuously during this 3-min interval with an O_2 sensor (Analytical Industries, Pomona, CA) attached to a data collection unit (Pasco, Roseville, CA) calibrated with atmospheric air. The final O_2 tension of each chamber was noted when the valves were closed and never exceeded 7.6 mmHg (~1% O_2).

Chelonia mydas eggs were collected from two ovipositing females in the field. These eggs were processed the same way as the freshwater turtle eggs described above. The eggshells were marked before being allocated to normoxic or hypoxic treatment groups. However, after eggs were allocated, they were transferred to a refrigerator and cooled for 10 h at below 10°C (Harry and Limpus 1989) to halt embryonic development. They were then transported by sea, land, and air in a 58-L sealed polystyrene container filled with expanded polystyrene pellets to the Monash University Animal Housing Facility, Melbourne. Transportation time was approximately 22 h, and the temperature in the container did not exceed 14°C. Once in the laboratory, the normoxic treatment and the 3-, 6-, and 9-day hypoxic treatment groups were placed in separate identical incubators at a constant temperature of 28°C. Treatment time was considered to begin when each group was placed in an incubator after transportation.

For all species, one egg from each clutch was dissected immediately after oviposition so that the stage of embryonic development could be determined with the staging criteria previously described for freshwater (Yntema 1968) and marine (Miller 1985) turtles. An additional egg from each clutch was dissected from each hypoxic chamber at the conclusion of the hypoxic treatment to identify whether embryonic development had progressed since the treatment began. One *C. mydas* egg was also opened after long-distance transport to ensure that development had not continued during shipping. All remaining unopened eggs were checked daily for formation of the characteristic white spot, considered to be the first visual sign that preovipositional arrest has broken and the egg is developing (Thompson 1985; Booth 2002). Observations continued until all eggs had either developed a white spot or were determined to be dead, in which case they were dissected to identify developmental stage at death.

O_2 Partial Pressure (Po_2) in the Oviducal Environment

Before measurement of O_2 partial pressure (Po_2) in the oviduct, gravid freshwater turtles were restrained in individual cotton bags and placed in 32°C incubators to increase body temperature for at least 1 h. Each turtle was then removed from the bag, weighed, and manually restrained in a right-lateral recumbent position with the head distal to the anesthetist. Anesthesia was induced by intravenous injection of alfaxalone (Alfaxan-CD RTU, 10 mg/mL alfaxalone) at a dose of 8 mg/kg via the jugular vein. Gravid *C. mydas* were obtained from the nesting beach and manually restrained before the procedure. Internal temperature was recorded in each female with a digital thermometer inserted into the cloaca.

The Po_2 in the oviduct (mmHg) was measured with an LAS-1 fiber-optic probe (1 mm in diameter, with sampling area 1 mm × 1 mm; Oxford-Optronix, Oxford, UK) attached to an OxyLite fluorescence lifetime oximeter set to record Po_2 at 1-s intervals (Oxford-Optronix). The OxyLite device was interfaced with a data acquisition system (Powerlab, AD Instruments, Sydney, Australia) connected to a laptop computer. Temperature compensation was performed manually, by setting the OxyLite temperature value to the internal temperature recorded for each turtle. The fiber-optic probe was threaded through an endoscope so that the tip of the probe was protruding approximately 1.5 mm from the distal aperture of the endoscope. Both the endoscope and the probe were directed through the cloacal opening of each turtle and into one of the oviducts. Images from the endoscope's video camera were displayed on a television monitor, which allowed confirmation that the O_2 measurement probe was inside the oviduct. A reading was accepted if the recorded Po_2 remained within ± 0.5 mmHg for at least 10 s. Measurements were attempted in both the right and left oviducts of each turtle.

O_2 Diffusion in Oviducal Secretions

Collection of Oviducal Fluid. Oviducal secretion samples were collected from 16 ovipositing green turtles. During oviposition, a 22 × 12.5-cm Ziploc bag was placed under the cloaca of each female after approximately 10 eggs had been laid into the nest chamber. Subsequent eggs were laid with their adhering oviducal secretions directly into the bag. Eggs were removed by hand as they were deposited

so that only the secretion remained in the bag. This continued until approximately 50 eggs had been laid into the bag. Secretion samples were then transferred to individual 5-mL vials, sealed, and refrigerated at 5°C for up to 48 h, until samples had been obtained from all females.

Measurement of O_2 Diffusion. From each of the 16 females, 1 mL of oviducal secretion was pooled, and the mixed secretion sample was redistributed evenly between eight 1.5-mL Eppendorf tubes. Five 1.5-mL Eppendorf tubes were also filled with 154 mM NaCl (saline) and acted as a control for this experiment. O_2 diffusion was measured in each of these eight secretion samples and five saline samples with a polarized Clark electrode (50-μm tip, Unisense, Århus, Denmark). The electrode was calibrated before use in a calibration chamber with pure O_2 (760 mmHg O_2) and pure nitrogen (0 mmHg O_2). Once calibrated, the probe was positioned, using micromanipulators, 2 mm below the surface of each secretion or saline sample. The probe was left standing in the sample for approximately 5 min, until Po_2 stabilized. Pure O_2 gas was then blown across the surface of the sample at a constant rate of 1 L/min from an outlet (a glass Pasteur pipette) positioned at a 30° angle 15 mm from the edge of the Eppendorf tube. The Po_2 at the probe tip was sampled at 1-s intervals for a 10-min period from the time the O_2 gas began blowing across the sample surface.

Data Analysis

The proportions of eggs forming white spots in the normoxic and hypoxic treatments were compared via Pearson's χ^2 test. The proportion of eggs forming white spots was calculated from the number of eggs remaining in each treatment group after some eggs had been dissected to identify their developmental stage. The time elapsed (days) from oviposition to white-spot formation was recorded, as was the time from the end of each hypoxic treatment until white-spot formation. An ANCOVA was used to identify whether the duration of hypoxia affected the period between oviposition and white-spot formation. These data were also subjected to ANOVA and Tukey's HSD tests to determine whether the duration of hypoxia affected the length of time between the end of the hypoxic treatment and white-spot formation.

Oviducal Po_2 measurements were analyzed with LabChart 7 (AD Instruments). For each oviduct, five consecutive values within the 10-s plateau period were averaged to obtain a single O_2 value for that oviduct. Paired *t*-tests were used to compare the left- and right-oviduct Po_2 values within each species.

The Po_2 values measured by the Clark electrode in oviducal fluid and saline were recorded at 1-s intervals (Universal Acquisition, University of Auckland). These values were binned into 1-min means for each fluid type for subsequent analysis. A linear mixed-effect model was run in R statistical software (R Development Core Team 2011) with the "nlme" package to identify whether the rate of diffusion differed between the secretion and saline samples. The rate of diffusion in each sample was treated as the response variable, sample type (secretion or saline) was the fixed effect, and sample number was the random effect. For all analyses, two-tailed $P \leq .05$ was considered statistically significant, and data presented are mean ± standard error.

Results

Effects of Hypoxia on Eggs in Preovipositional Arrest

In total, 74 eggs from 12 *Chelodina oblonga*, 68 eggs from nine *Chelodina longicollis*, 76 eggs from six *Emydura macquarii*, and 82 eggs from two *Chelonia mydas* females were used in the study. The distribution of eggs across treatment groups for each species is shown in table 1.

Preovipositional developmental arrest was prolonged in a hypoxic environment. No eggs developed white spots (the first visual sign that preovipositional arrest has broken and the egg is developing) during the 3-, 6-, or 9-day hypoxic treatments (fig. 1). This resulted in a significant increase in the time from oviposition to formation of a white spot as the period of hypoxic incubation increased from 0 to 3, 6, and 9 days for *C. oblonga* ($df = 23$, $F = 16.46$, $P = .001$, slope = 1.89), *C. longicollis* ($df = 36$, $F = 30.85$, $P < .001$, slope = 1.14), *E. macquarii* ($df = 54$, $F = 604.5$, $P < .001$, slope = 1.13), and *C. mydas* ($df = 63$, $F = 4{,}366$, $P < .001$, slope = 1.53; table 1). In the normoxic control, *C. mydas* eggs took an average 1.3 ± 0.1 days to form white spots, *E. macquarii* eggs took 2.1 ± 0.2 days, and *C. longicollis* and *C. oblonga* eggs took 2.6 ± 0.8 and 11.5 ± 1.7 days, respectively.

All freshwater and marine turtle eggs dissected at oviposition and after 3, 6, and 9 days in hypoxia were at Yntema's (1968) stage 0 and Miller's (1985) stage 6 of development, respectively, indicating that developmental arrest had continued during the treatment. Successful recommencement of development and the formation of white spots at the conclusion of each treatment were seen when eggs were subsequently exposed to normoxia. However, the proportion of eggs that successfully recommenced development differed significantly between treatments for both *C. oblonga* ($df = 3$, $\chi^2 = 15.14$, $P < .001$) and *C. mydas* ($df = 3$, $\chi^2 = 20.60$, $P < .001$), with the number of eggs surviving the hypoxia generally declining with increased duration in hypoxia. The proportion of eggs dying increased from 21.1% in the normoxic control to 80% in

Table 1: Effects of hypoxia on egg development

	Control (normoxia)	Hypoxia, 3-day treatment	Hypoxia, 6-day treatment	Hypoxia, 9-day treatment
Chelodina oblonga:				
No. of eggs in treatment (*n*)	24	15	15	20
No. of eggs dissected after treatment[a]	5	6	5	5
No. (%) of remaining eggs forming white spot[b]	15 (78.9)	4 (44.4)	2 (20.0)	3 (20.0)
No. (%) of eggs failing to begin development[c]	4 (21.1)	5 (55.6)	8 (80.0)	12 (80.0)
Mean days to white spot after treatment (±SEM)	11.5 ± 1.7	13.0 ± 1.5	15.0 ± 1.0	20.3 ± 9.1
Chelodina longicollis:				
No. of eggs in treatment (*n*)	13	17	18	20
No. of eggs dissected after treatment[a]	5	5	5	5
No. (%) of remaining eggs forming white spot[b]	7 (87.5)	10 (83.3)	10 (76.9)	10 (66.7)
No. (%) of eggs failing to begin development[c]	1 (12.5)	2 (16.7)	3 (23.1)	5 (33.3)
Mean days to white spot after treatment (±SEM)	2.6 ± .8	5.9 ± 2.2	3.4 ± .4	5.1 ± .5
Emydura macquarii:				
No. of eggs in treatment (*n*)	19	22	18	17
No. of eggs dissected after treatment[a]	4	4	3	2
No. (%) of remaining eggs forming white spot[b]	15 (100)	17 (94.4)	14 (93.3)	11 (73.3)
No. (%) of eggs failing to begin development[c]	0 (0)	1 (5.6)	1 (6.7)	4 (27.7)
Mean days to white spot after treatment (±SEM)	2.1 ± .2	2.2 ± .3	3.0 ± .3	3.1 ± .3
Chelonia mydas:				
No. of eggs in treatment (*n*)	35	16	15	16
No. of eggs dissected after treatment[a]	3	1	1	1
No. (%) of remaining eggs forming white spot[b]	32 (100)	14 (93.3)	7 (50.0)	11 (73.3)
No. (%) of eggs failing to begin development[c]	0 (0)	1 (6.7)	7 (50.0)	4 (26.7)
Mean days to white spot after treatment (±SEM)	1.3 ± .1	3.0 ± .0	4.1 ± .3	6.1 ± .4

[a] Total number of eggs dissected in each treatment to identify the stage of embryonic development.
[b] Total number of eggs forming white spots in each treatment, not including the eggs that were dissected to identify developmental stage.
[c] Total number of eggs that failed to develop a white spot and were considered dead.

the 9-day hypoxic treatment for *C. oblonga* and from 0% to 26.7% in the normoxic and 9-day hypoxic treatments, respectively, for *C. mydas* (table 1). No significant difference was observed in the proportion of eggs dying in the normoxic or hypoxic treatments for *C. longicollis* (df = 3, $\chi^2 = 1.68$, $P = 0.64$) or *E. macquarii* (df = 3, $\chi^2 = 7.17$, $P = .07$). However, a trend toward an increase in the proportion of eggs dying with increased duration in hypoxia was observed in both cases. An increase from 12.5% mortality in the normoxic control to 33.3% in the 9-day hypoxic treatment occurred for *C. longicollis*; for *C. mydas*, the corresponding increase was from 0% to 27.7% (table 1).

The period of time it took to resume development and form a white spot after each hypoxic treatment finished generally exceeded the time actually spent in hypoxia. In addition, the time to white-spot formation generally became longer with increased duration in hypoxia, relative to that in the normoxic control. There was a significant increase in the number of days to formation of white spot as the period of hypoxia progressed from 0 days in the normoxic control to 3, 6, and 9 days for *C. mydas* (df = 3, $F = 176.02$, $P < .001$). A significant difference in the time to white-spot development was also identified between the eggs in the normoxic control and those in the 3-, 6-, and 9-day hypoxic treatments for *E. macquarii* (df = 3, $F = 3.02$, $P = .04$), although a post hoc Tukey's HSD test did not identify any specific treatment as significantly different from another. The duration of hypoxia did not significantly affect the time from exposure to normoxia to white-spot formation in eggs of *C. longicollis* (df = 3, $F = 1.32$, $P = .29$) or *C. oblonga* (df = 3, $F = 1.19$, $P = .34$; table 1).

O_2 Partial Pressure (Po_2) in the Oviducal Environment

The Po_2 was measured in both left and right oviducts for four *C. oblonga*, seven *C. longicollis*, and three *E. macquarii* turtles, in addition to 11 single-oviduct readings (three right and eight left) for *C. mydas* turtles. Three single right-oviduct measurements and one single left-oviduct measurement were obtained from an additional four *C. oblonga*. Paired *t*-tests were used to compare the left- and right-oviduct Po_2 values within each species (except the marine turtles, in which only one oviduct was sampled in each female). Results indicated that the Po_2 measured in

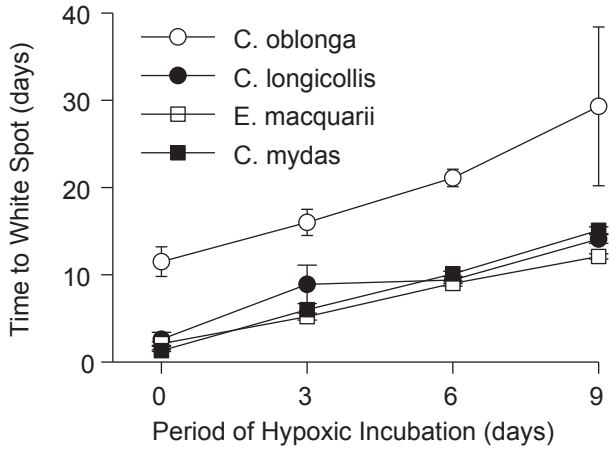

Figure 1: Mean time (days) from oviposition to white-spot formation of eggs in normoxic (0 days) and hypoxic (3, 6, or 9 days) treatments ± SEM.

the left oviduct did not differ significantly from that in the right oviduct in *C. oblonga* (df = 3, $t = 2.50$, $P = .09$), *C. longicollis* (df = 6, $t = 0.98$, $P = .36$), or *E. macquarii* (df = 2, $t = 0.39$, $P = .73$), so the four single-oviduct readings for *C. oblonga* were included in the sample used for calculation of the between-animal mean value for that species. Mean oviductal Po_2 was 5.9 ± 2.5 mmHg in *C. oblonga* ($n = 8$), 1.6 ± 1.2 mmHg in *C. longicollis* ($n = 7$), 5.3 ± 2.1 mmHg in *E. macquarii* ($n = 3$), and 2.9 ± 1.4 mmHg in *C. mydas* ($n = 11$; fig. 2).

O_2 Diffusion in Oviductal Secretions

The mean Po_2 measured at the beginning of the experiment was significantly lower in oviductal secretions (84.6 ± 3.3 mmHg; range 72.4–99.8 mmHg) than in saline (126.3 ± 12.6 mmHg; range 86.6–160.4 mmHg; df = 1, $F = 15.6$, $P = .002$; fig. 3). Exposure of the sample to a stream of 100% O_2 resulted in a progressive increase in Po_2 in all samples. However, the rate at which Po_2 increased over the 10 min of exposure to 100% O_2 was significantly greater for saline (14.2 ± 2.1 mmHg/min) than for the oviductal secretion (1.9 ± 0.6 mmHg/min; df = 1, $F = 64.3$, $P = .001$). Furthermore, the mean Po_2 after 10 min of exposure to 100% O_2 was significantly lower in the oviductal-secretion samples (103.5 ± 5.8 mmHg; range 87.1–134.0 mmHg) than the final measurement recorded in the saline (268.3 ± 30.2 mmHg; range 216.4–380.4 mmHg; df = 1, $F = 45.4$, $P < .001$; fig. 3).

Discussion

Our results suggest that the restricted diffusive ability of oviductal secretions possibly creates or contributes to an extremely hypoxic oviductal environment and limits embryonic development in turtles. We showed that hypoxia maintained preovipositional arrest after oviposition and that subsequent exposure to increased O_2 partial pressure reinitiated active development. A similar phenomenon has been observed in natural nests of the north Australian long-necked turtle (*Chelodina rugosa*), which lays eggs underwater in flooded billabongs; its eggs do not begin developing until the nests dry out and O_2 tension increases (Kennett et al. 1993). On the basis of our collective observations, it is plausible to hypothesize that restricted O_2 availability in the reptilian oviductal environment limits embryonic development and may therefore constrain the evolution of viviparity in those animals.

Marine and freshwater turtle embryos typically arrest during early development stages—stage 6 of 31 for marine embryos (Miller 1985) and stage 0 of 26 for freshwater embryos (Ewert 1985)—whereas lizard embryos typically arrest at around stage 30 of a 40-stage developmental chronology (Andrews and Mathies 2000). Mean Po_2 in turtle oviducts is substantially lower than estimates in sceloporine lizards, which is suggested to exceed 40 mmHg (Parker and Andrews 2006). It is therefore plausible that turtle embryos become arrested during earlier stages than lizard embryos because lower oviductal O_2 tensions limit development earlier in the developmental schedule (Andrews and Mathies 2000; Parker and Andrews 2006). Therefore, we hypothesize that the onset of preovipositional arrests

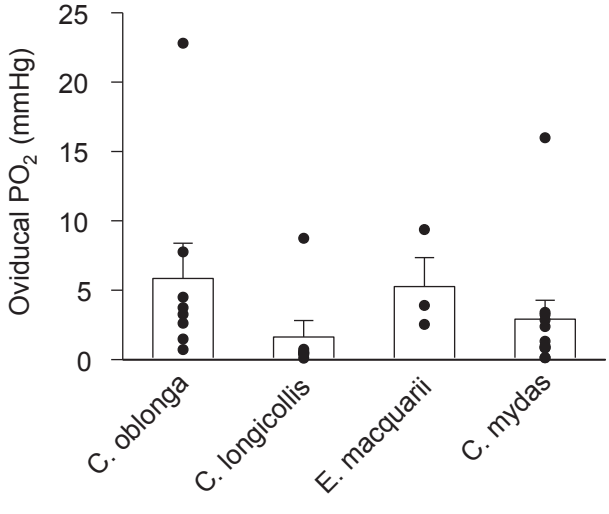

Figure 2: Partial pressure of oxygen (Po_2) in oviducts of gravid *Chelodina oblonga* ($n = 8$), *Chelodina longicollis* ($n = 7$), *Emydura macquarii* ($n = 3$), and *Chelonia mydas* ($n = 11$) + SEM.

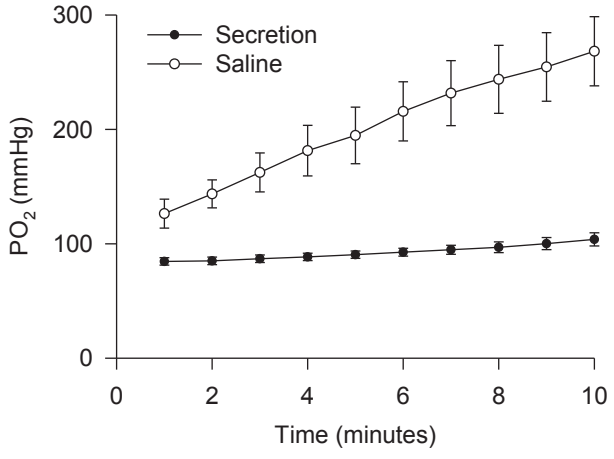

Figure 3: Comparison of rates of oxygen diffusion over 10 min in saline ($n = 5$) and in oviducal secretions ($n = 8$) of the green turtle, *Chelonia mydas*. Each point represents the between-samples mean of 1-min averages ± SEM.

occurs when embryonic oxygen demand exceeds oxygen availability in the oviduct. However, further investigations are needed to quantify embryonic oxygen demand in utero and the simultaneous availability of oxygen in the oviduct to confirm whether this is the case.

What mechanisms could limit the availability of O_2 to embryos in the reptilian oviduct? Full calcification of turtle and squamate eggs directly precedes the onset of preovipositional arrest and may prevent the rate of respiratory gas exchange that would be necessary for further embryonic development (Packard et al. 1977; Guillette 1982; Palmer et al. 1993; Heulin et al. 2002). A reduction in eggshell thickness has been associated with extended egg retention and advanced embryonic development in reptiles (Mathies and Andrews 1995; Heulin et al. 2002), presumably because the availability of O_2 to the embryo is enhanced relative to that in eggs with thicker shells. However, the eggshell provides a source of calcium for developing turtle embryos, and reducing the degree of eggshell calcification in order to achieve greater O_2 exchange may decrease embryo fitness (Andrews and Mathies 2000; Bilinski et al. 2001).

In addition, in order for embryogenesis to occur in turtle and crocodile eggs, the vitelline and inner shell membranes must adhere to each other ("chalk") to facilitate the gas diffusion necessary for subsequent embryonic development. Chalking of turtle and crocodile eggs does not occur until after oviposition, and as they are incapable of preovipositional arrest, crocodiles need to lay their eggs immediately or developmental deformities arise, probably because of compromised O_2 availability (Ferguson 1985). It is unknown why chelonians are capable of arresting development in a hypoxic environment and crocodilians are not. Chalking of turtle and crocodile eggs does not occur until after oviposition, presumably because if eggs are laid after membrane adhesion, membrane rupture and embryonic mortality may result from the physical movement and jostling of eggs during oviposition (Limpus et al. 1979; Andrews and Mathies 2000). Furthermore, the presence of viscous oviducal secretions surrounding the egg and filling its pores is likely to inhibit formation of a respiratory surface that allows efficient gas diffusion (Andrews and Mathies 2000).

Previous work implicated respiratory activity of the oviduct and the chorioallantoic membrane of the embryo, in addition to the O_2-binding properties of embryonic blood, as the primary factors mediating gas exchange in the oviduct (Parker et al. 2004; Parker and Andrews 2006). However, preovipositional arrest occurs before development of the chorioallantoic membrane and blood circulation in turtles (Ewert 1985; Miller 1985), suggesting that these factors are not controlling or limiting gas exchange in the oviduct in this group. Our results provide strong evidence that oviducal secretions limit O_2 diffusion, a property that may be the key to understanding how hypoxia arises and limits development in the oviduct.

It is thought that evolution of reptilian viviparity required increasingly extended periods of oviducal egg retention, coupled with advanced stages of embryonic development in the oviduct (Shine 1985). The olive ridley marine turtle (*Lepidochelys olivacea*) is capable of retaining eggs for up to 63 days during prolonged internesting periods, but embryos are always laid as gastrulae despite this extended egg retention (Plotkin et al. 1997). Evidently, extended egg retention alone will not necessarily lead to the evolution of viviparity unless selection for physiological traits that facilitate and enhance O_2 availability also occur simultaneously (Andrews and Mathies 2000).

Our findings indicate that extended periods of preovipositional arrest in response to hypoxia (simulating extended oviducal egg retention) significantly reduce survival of *Chelodina oblonga* and *Chelonia mydas* embryos, at least under our experimental conditions. Developmental deformities and death of turtle embryos have been linked to periods of extended egg retention and prolonged preovipositional arrest (Ewert 1985). Embryonic death of sceloporine lizard embryos also increases during periods of experimentally induced hypoxia (Parker and Andrews 2006). Therefore, it is possible that extended periods in hypoxia can negatively affect embryonic survival, thus selecting for shorter egg retention intervals that ultimately prevent the evolution of viviparity (Rafferty et al. 2011). Extended oviducal egg retention decreases postovipositional embryonic survival early in development in naturally nesting leatherback turtles (Rafferty et al. 2011).

However, it is also possible that increased mortality in laboratory tests is due to artificial manipulation of the incubation environment, because no significant difference was observed in the proportion of eggs dying in the normoxic or hypoxic treatments for *Chelodina longicollis* or *Emydura macquarii* even though they were incubated under the same conditions as the two other species.

Our study shows that oviducal hypoxia induces and maintains preovipositional arrest in turtle embryos. It is possible that the production of oviducal secretions that greatly inhibit oviducal O_2 diffusion is an important mechanism that promotes hypoxia in the reptile oviducal environment. Very little is known about the properties and roles that oviducal secretions play during embryonic development, and understanding such processes may allow a concrete conclusion to be drawn about the evolution of viviparity. Therefore, future research needs to consider why turtles have retained the oviducal secretions that inhibit oviducal O_2 diffusion and why this controlling factor has not been modified in turtles as it may have been in other reptiles. In order to do so, it would be useful to researchers to take a comparative approach to their investigations and also to consider squamate reptiles in addition to aquatic species, including teleost fish, sharks, and rays that are viviparous.

The results of this study suggest that oviducal hypoxia may constrain the evolutionary transition between reproductive modes and could possibly preclude the evolution of viviparity in amniote reptiles. In addition, extended periods of egg retention may not lead to viviparity unless physiological features that enhance O_2 availability in developing embryos also evolve concurrently.

Acknowledgments

We thank the Holsworth Wildlife Foundation and Monash University for financial support. Thanks also to D. Chapple, P. Comber, D. Dowling, G. Eppel, J. Giles, G. Kuchling, B. Lees, B. Prince, D. Stockheld, and B. Wong for their valuable help and advice. This study was made possible with support offered by B. Tormey, J. Van Rijn, Austvet, Zoos Victoria, and the staffs of the Healesville Sanctuary Wildlife Health Centre and the Heron Island Research Station. Research was conducted under scientific permits issued by the Queensland Environmental Protection Agency (WITK0906210 and WISP08063210), the Victoria Department of Sustainability and the Environment (10005293), and the Western Australia Department of Environment and Conservation (SF007435; CE002893). It was approved by the Biological Sciences Animal Ethics Committee of Monash University (BSCI/2009/23 and BSCI/2009/28).

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Associate Editor: Tony D. Williams
Editor: Mark A. McPeek

Chelonia mydas (green turtle) eggs in a nest, taken during nesting. Photograph by T. Franciscus Scheelings.