When Is Embryonic Arrest Broken in Turtle Eggs?

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ABSTRACT

Turtle embryos enter a state of arrested development in the oviduct, allowing the mother greater flexibility in her reproductive schedule. Development recommences once eggs transition from the hypoxic oviduct to the normoxic nest. Significant mortality can occur if turtle eggs are moved between 12 h and 20 d after oviposition, and this is linked to the recommencement of embryonic development. To better understand the timing of developmental arrest and to determine how movement-induced mortality might be avoided, we determined the latency (i.e., time elapsed since oviposition) to recommencement of development following oviposition by exposing the eggs of green turtles (*Chelonia mydas*) to hypoxia (oxygen tension <8 mmHg) for 3 d, commencing 30 min to 48 h after oviposition. Embryonic development—including development of the characteristic opaque white spot on the eggshell—was halted by hypoxic incubation. When the delay before hypoxic incubation was 12 h or less, hatching success did not differ from a control group. If the hypoxic treatment began after 16 h or more in normoxia, then all embryos died. Thus, by returning eggs to a hypoxic environment before they have broken from arrest (i.e., within 12 h of oviposition), it is possible to extend embryonic arrest for at least 3 d, with no apparent detriment to hatching success. Therefore, hypoxic incubation may provide a new approach for avoidance of movement-induced mortality when conservation or research efforts require the relocation of eggs. Our findings also suggest that movement-induced mortality may have constrained the evolution of viviparity in turtles.

Keywords: turtle, embryo, arrested development, preovipositional arrest, reproduction, oxygen.

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Introduction

Turtle ecology and evolutionary history is greatly influenced by a single physiological trait: preovipositional embryonic arrest. The arrest within the egg occurs in all turtle species (Ewert 1985) and plays a critical role in their reproductive success and life history (Ewert 1991; Rafferty and Reina 2012). Turtle embryos progress to an early stage of embryonic development within the mother—a mid- or late gastrulae for marine and freshwater turtles, respectively—before they then enter preovipositional arrest (Ewert 1985; Miller 1985). The arrest maintains the embryos at this stage of development, with no active cellular division or growth occurring (Rafferty and Reina 2012).

Preovipositional embryonic arrest offers many advantages. The mother is able to delay oviposition by days (Rafferty et al. 2011), weeks (Plotkin et al. 1997), or even months (Kennett et al. 1993; Buhlmann et al. 1995) until favorable conditions arise, therefore having greater flexibility in her reproductive schedule. Further, it ensures that all the embryos are at the same developmental stage when they are laid. This facilitates synchronous development within a nest of eggs that may have been ovulated up to 48 h apart (Licht 1980, 1982; Licht et al. 1982; Owens and Morris 1985). In turn, this enables synchronous hatching to occur to (1) avoid predation (Spencer et al. 2001; Santos et al. 2016) and (2) decrease the energetic cost of nest escape (Rusli et al. 2016). Finally, because the preovipositional arrest pauses development before the embryonic membranes have attached to the egg shell membranes, the eggs are protected from movement-induced mortality when they are dropped into the nest during oviposition (Ewert 1991; Rafferty et al. 2013; Rings et al. 2015).

Although we know of these important implications of preovipositional arrest for turtle life history and reproductive success, we still have limited knowledge as to how the arrest functions. A clue lies in the finding of Kennett et al. (1993) that freshwater turtle eggs laid under water are maintained in embryonic arrest after oviposition because of a lack of available oxygen, suggesting an important role of oxygen in the control of development. Improving our understanding of embryonic arrest will better inform our knowledge of the evolutionary and ecological physiology of turtles, which may lead to improved conservation outcomes. At least for marine turtles, conservation has primarily been focused on the nesting and incubation phases of their life history (Hamann et al. 2010), so better understanding of preovipositional embryonic arrest will allow us to inform management decisions concerning the relocation and incubation of turtle eggs.

One recent advance was the discovery by Rafferty et al. (2013) that when the egg moves from the hypoxic environment of the oviduct into the normoxic environment of the nest, the change in the partial pressure of oxygen is the trigger for the embryo

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to break preovipositional arrest (i.e., to start developing again). Further, it has been shown that placing eggs back into a hypoxic environment immediately after laying extends the embryonic arrest for several days until the eggs are placed back into normoxia (Kennett et al. 1993; Rafferty et al. 2013; Rings et al. 2015). This mechanism—by which the hypoxic maternal oviduct prevents extended embryonic development before laying—allows turtles to have greater plasticity in the timing of oviposition. Rafferty and Reina (2012) speculated that the hypoxia in the mother's oviduct may upregulate insulin-like growth factor binding protein, which binds to and suppresses insulin-like growth factors, thereby preventing development and growth. When the partial pressure of oxygen in the eggs' environment increases after eggs are oviposited, the suppression of insulin-like growth factors would then be greatly reduced, causing the embryo to continue development (Rafferty and Reina 2012).

Following oviposition, the formation of an opaque white spot on the upper surface of turtle and crocodile eggs is the first sign that active embryonic development is occurring (Thompson 1985; Webb et al. 1987*b*) and is typically seen in the first few days after oviposition (Ewert 1991). The white spot is a result of the vitelline embryonic membrane migrating through the albumin and attaching to the egg shell membranes, followed by drying of the outer layer of the egg shell where these membranes have fused (Thompson 1985; Webb et al. 1987*b*). The white spot then functions as a respiratory surface for the developing embryo, allowing greater gas exchange to occur. The spot continues to spread as the embryo develops, eventually encompassing the whole egg as the embryo's metabolic demands increase (Deeming and Thompson 1991; Thompson 1993). If a turtle or crocodile egg is turned or vigorously moved while the embryo is still in the first 12 h to 20 d of development, these fused membranes can easily rupture, resulting in the death of the embryo (Limpus et al. 1979; Webb et al. 1987*a*, 1987*b*). All turtle and crocodile eggs are known to experience this movement-induced mortality before the embryo and its membranes have grown large enough to rotate freely within the egg with no damage (Deeming 1991). Because the increase in the partial pressure of oxygen at the time of laying is the trigger that breaks preovipositional arrest (Rafferty et al. 2013; Rings et al. 2015), we could expect that artificially maintaining eggs in hypoxia after laying will protect them from movement-induced mortality because it would delay development and adhesion of embryonic membranes.

Rafferty et al. (2013) and Rings et al. (2015) showed that placing eggs of marine and freshwater turtles into hypoxia within 10 min of them leaving the cloaca extends embryonic arrest. However, the subsequent hatching success of these eggs was relatively low. Kennett et al. (1993) achieved relatively good hatching success in two freshwater species (*Chelodina rugosa* and *Chelodina longicollis*) when eggs were laid underwater and kept submerged. Knowing the duration of exposure to normoxia required to break preovipositional embryonic arrest is the first step in understanding whether it is possible to use hypoxia to artificially maintain arrest after laying. If arrest is impacted on by even a very short period of increased oxygen availability, use of artificial hypoxia to safely (i.e., without a reduction in hatching success) extend arrest after oviposition may not be feasible. Furthermore, it is not known whether early stage embryos are able to survive being placed back into hypoxia once they have broken from preovipositional arrest. Kennett et al. (1993) was unable to observe reentry of *C. rugosa* and *C. longicollis* eggs into arrest at 10 and 20 d after oviposition, but we might expect that embryonic development was relatively advanced at that point, likely between Yntema's (1968) stages 9 and 20 of development (Ewert 1985). It seems possible that reentry into hypoxia may be possible at a much earlier stage. Any ability of embryos to reenter embryonic arrest would be of ecological and evolutionary significance. One example may be the potential during the early period of nest incubation to survive periods of unfavorable environmental conditions, such as nest inundation by heavy rains or extreme tidal events. We hypothesized that the breaking of arrest occurs approximately 12 h after oviposition, because this is when the embryo generally becomes sensitive to movement-induced mortality (Limpus et al. 1979; Parmenter 1980).

To better understand the process of breakage of embryonic arrest and the plasticity of this process, we investigated the roles of hypoxia, normoxia, and elapsed time in the development of green turtle (*Chelonia mydas*) embryos. For the purposes of this study, we considered green turtle embryos as a model for all turtle species. Embryos from all species of turtle—both freshwater and marine—undergo preovipositional development arrest. In addition, stages of development and developmental schedules are well conserved within turtles, especially in the early stages of development when embryos undergo preovipositional embryonic arrest (Ewert 1985; Miller 1985). Furthermore, green turtles lay clutches with some of the largest numbers of eggs out of all turtle species, and they are one of the most abundant species of turtle in the world. Our aims were (1) to identify the latency of breakage of embryonic arrest after oviposition and (2) to test the ability of embryos to reenter embryonic arrest and survive hypoxia once they recommence development. Identifying the precise timing of these events will help better inform turtle researchers and conservationists regarding the time window for safe transportation of eggs and potentially provide a new method for doing so. Further, it improves our understanding of this fascinating and important physiological adaptation that allows greater control over reproductive timing, influencing turtle life history, ecology, and evolution.

Methods

Regulatory Approval

All experimental procedures were approved by Monash University's School of Biological Sciences Animal Ethics Committee (approval BSCI/2014/23), in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The research was conducted under a scientific permit issued by the Queensland Department of Environment and Heritage Protection (WITK15232014).

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Egg Collection

Eggs ($N = 364$) of green turtles (*Chelonia mydas* [Linnaeus]) were collected from ovipositing females ($N = 6$) on two nights at Heron Island, Great Barrier Reef, Australia. On January 29, 2015, between 2000 and 2300 hours, 181 eggs were collected carefully by gloved hand from three females. A further 183 eggs were collected in the same manner from three more females on the night of February 3, 2015, between 1900 and 2200 hours. The approximate time (±1 min) of oviposition for each egg was recorded. Eggs were individually numbered with a soft pencil on their uppermost surface as they were collected. Once the last egg had been collected from each female, the eggs from that individual were carried by hand in buckets a short distance (<700 m, 5–10-min walk) to the laboratory at the Heron Island Research Station. The mass (g) and diameter (mm) of each egg were measured once the eggs arrived at the laboratory.

Hypoxic Treatments to Assess Embryonic Arrest

Eggs from each clutch were randomly allocated to one of 10 treatments. One group of eggs from each clutch served as a control and were incubated in normoxia (~21% O_2) for their entire developmental period ($N = 40$). The other nine groups of eggs were first incubated in normoxia (in sand) and then placed into hypoxia (~1% O_2 in a Perspex chamber) at 0.5, 1, 2, 4, 8, 12, 16, 24, and 48 h after oviposition ($n = 36$ in each case). These eggs remained under hypoxic conditions for 3 d before being incubated in normoxia again. For example, eggs in the 0.5-h group were in normoxia for 30 min after oviposition, then in hypoxia until 3 d and 30 min after oviposition, and then in normoxia until hatching, while eggs in the 1-h group were in normoxia for 1 h after oviposition, then hypoxia until 3 d and 1 h after oviposition, and then in normoxia for the rest of development, and so on for the other groups.

Eggs were maintained in hypoxia by placing them in airtight Perspex containers (Resi-Plex Plastics, Victoria) using established techniques (Rafferty et al. 2013; Rings et al. 2015). The eggs were placed on a wire mesh, allowing them to sit above approximately 10 mL of water at the base of each box. Each container had an inflow and an outflow valve at opposite ends of the box, and industrial-grade 100% nitrogen gas (BOC, North Ryde, New South Wales) was humidified by pumping it through a water chamber and then into the Perspex container at a flow rate of 8 L min^{-1}. The partial pressure of oxygen (P_{O_2}) in the gas leaving the outflow valve of each box was monitored using an oxygen sensor (Analytical Industries, Pomona, CA) and a data collection device (Pasco, Roseville, CA) to ensure that the atmosphere in the container had reached approximately 1% oxygen v/v (P_{O_2} ~8 mmHg). The containers were then sealed and placed in incubators (GQF HovaBator model 1632; Grandview Management, Baldivis, Western Australia) set to 28°C. The containers were regassed approximately every 24 h over the 3-d treatment period. During regassing, the P_{O_2} was monitored as the gas flowed through the chamber to ensure that the P_{O_2} had remained stable at approximately 8 mmHg.

Once the eggs arrived at the laboratory, two eggs from the control group were opened to visually identify the embryonic stage at oviposition. A further two eggs were opened from each hypoxic treatment group immediately before the group of eggs was placed into hypoxia, with another two opened immediately after the hypoxic treatment concluded. The stage of development of these embryos was determined according to Miller's (1985) 31-stage developmental chronology for marine turtles.

Egg Incubation

Other than when eggs were in hypoxia, they were exposed to atmospheric oxygen and placed in sand (~7% moisture content by mass) in GQF HovaBator incubators set to 28.0°C. Eggs in incubators were visually checked three times per day for formation of an opaque white spot on the shell as the first visible sign that embryonic development was occurring. Once a white spot had formed, growth of the white spot on the eggshell was recorded using calipers to measure the maximum diameter of the white spot to the nearest millimeter. Eggs that formed a white spot before being placed into hypoxia did not have their white spot growth measured while they were in hypoxia because this would have required opening the container and allowing atmospheric air to enter. The white spot for these eggs was measured both immediately before the egg was placed in hypoxia and immediately following removal of the egg from hypoxia. Any eggs that showed visible signs of embryonic death (such as green discoloration or the presence of fungus) were removed from the incubators and also staged according to Miller's (1985) guide.

Once all eggs had completed their hypoxic treatment and had formed white spots, they were carefully removed from their incubators and transported a short distance (<300 m) back to the nesting beach. Nest cavities were excavated by hand to a total depth of 60 cm in the natural shape of the nests of *C. mydas*. At the time of burial, eggs from the first and second nights of egg collection were a total of 14 and 9 d after oviposition, respectively. Before burial, all eggs were individually numbered on two additional locations on the egg, using a soft pencil to ensure that each egg or eggshell could be identified once the hatchlings emerged and the nest was excavated. Eggs from the various treatment groups were randomly placed at different depths within the nest cavities.

Excavation of Nests

Approximately 2 d after hatchlings had emerged, each nest was excavated to determine hatching success. Unhatched eggs were transported to the laboratory for identification and staging of the embryo. Hatched eggshells were carefully examined to identify the individual number of each successfully hatched egg. Unhatched eggs were opened in the laboratory, and the stage of the embryo was identified according to Leslie et al.'s (1996) field-staging method. This method classifies Miller's (1985) 31-stage developmental chronology into four broader

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classifications of the stage of development, as described in detail by Rafferty et al. (2011).

Statistical Analysis

ANOVA (with treatment as the independent factor and clutch identity as a random blocking factor) and Tukey's honest significant difference (HSD) tests were used to determine between-group differences in the mass and diameter of eggs, the total time to formation of a white spot, aerobic time (total development time excluding time in hypoxia) to formation of a white spot, and growth rate of the white spot after removal of the egg from hypoxia. Data were checked for normality and homogeneity of variances for each ANOVA test performed.

Hatching success was defined as the number of eggs to hatch divided by the total number of eggs, excluding those that had been deliberately opened and staged. Cochran-Mantel-Haenszel (CMH) tests with Bonferroni corrections for pairwise comparisons were used to assess between-group and clutch variation in hatching success and the proportion of white spots that formed before exposure to hypoxia. Post hoc analysis of the CMH tests were conducted using χ^2 tests with Bonferroni corrections for pairwise comparisons with the independent variable of interest being the treatment group. A χ^2 test was also used to compare hatching success between eggs from the two collection nights. There was a difference in the proportion of eggs that hatched from the two collection nights. This may have been because the eggs from the second night of collection were at an earlier stage of development (5 d younger) when they were relocated to the nests on the beach, possibly making them more susceptible to movement-induced mortality. Importantly, data regarding morphology of the egg, development, and growth of the white spot were collected before moving the eggs. Thus, we included data from eggs collected on both nights for these analyses. However, because of our concerns about the impact of movement-induced mortality, eggs collected on the second night were excluded from the analysis of hatching success. CMH tests with Bonferroni corrections for pairwise comparisons were also used to examine between-group differences in the proportion of embryos from the first collection night that died at each developmental field stage (Leslie et al.'s [1996] four stages of developmental chronology) between the treatments with high hatching success (greater than 70%; control, 0.5-, 1-, 2-, 4-, 8-, and 12-h treatment groups) and the treatments with low hatching success (0%; 16-, 24-, 48-h treatment groups). Post hoc analysis of the Cochran-Mantel-Haenszel tests was conducted using χ^2 tests with Bonferroni corrections for pairwise comparisons for the independent variable of treatment group. All analyses were performed using R software (R Development Core Team 2013). All values are means $\pm$ SE unless otherwise stated. Two-tailed $P \leq 0.05$ was considered statistically significant.

Results

Egg Morphology, Development, and White Spot Formation

There was no significant variation across the treatments in the mass (g; $F_{9,43} = 10.50$, $P = 0.06$) and diameter (mm; $F_{9,43} = 1.06$, $P = 0.41$) of eggs at collection (table 1). There was also little between-group variation in the proportion of eggs to form a white spot. Indeed, all eggs formed a white spot except for one egg in the control group and one egg in the 12-h treatment. No eggs in any of the treatment groups formed white spots while in hypoxia. There was no variation in the aerobic time taken to form a white spot ($F_{9,43} = 1.50$, $P = 0.18$; fig. 1). The latency to white spot formation across all groups was 39 $\pm$ 1 cumulative hours in normoxia ($n = 31$–37). The latency to formation of a white spot—when including time spent in hypoxia—differed markedly among treatment groups ($F_{9,43} = 20.16$, $P < 0.0001$) in a systematic manner (fig. 1). That is, white spot formation was delayed by approximately 72 h (i.e., the duration of the hypoxic incubation) in all groups in which the elapsed time before hypoxic incubation was 16 h or less. There was also systematic variation in the proportion of eggs in each treatment that formed white spots before hypoxia ($\chi^2_{CMH} = 15.73$, df = 5, $P < 0.01$; fig. 1). That is, no eggs formed white spots before hypoxia when the delay before hypoxic incubation was 16 h or less. In contrast, 47% formed white spots before hypoxia when the delay was 24 h, and 97% formed white spots before hypoxia when the delay was 48 h.

Growth rate of the white spot (mm/h) was affected by exposure to hypoxia ($F_{9,41} = 10.46$, $P < 0.0001$; fig. 2). In eggs from the 0.5-, 2-, 4-, 8-, 12-, 16-, and 24-h treatments, growth rate of the white spot after hypoxia was significantly slower than the growth rate for the control eggs. Growth rate after hypoxia was even lower in the eggs in the 48-h treatment group. In this treatment group, growth rate of the white spot slowed from 0.45 $\pm$ 0.03 mm/h before hypoxia to only 0.02 $\pm$ 0.00 mm/h while the eggs were in hypoxia.

There was no detectable development of embryos while in hypoxia, as ascertained from opening and staging eggs from each treatment before and after hypoxia (table 2). Eggs opened before entering hypoxia were mostly at the same stage of development as eggs from the same treatment that were opened once they were removed from hypoxia (table 2). There was no detectable growth of embryos until after 12 h of exposure to normoxia. The most developed eggs that were staged were from the 48-h treatment, in which embryos were found to be at Miller's stages 9 or 10 (table 2).

Hatching Success

There was significant variation in hatching success between the treatments among the different clutches ($\chi^2_{CMH} = 130.02$, df = 5, $P < 0.0001$).The difference between clutches was due to the significant difference between the hatching success depending on the night of collection ($\chi^2 = 97.66$, df = 1, $P < 0.0001$). That is, many more eggs hatched from the clutches that were collected on the first night (57% of 181 unopened eggs) compared with those that were collected on the second night (5% of 145 unopened eggs). Even after excluding eggs from the second night of collection, the between-group variation in hatching success was statistically significant ($\chi^2 = 107.20$, df = 9, $P < 0.0001$; fig. 3). Within the eggs from the first collection, hatching

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Table 1: Average mass and diameter of green turtle eggs from each treatment

	Control	0.5 h	1 h	2 h	4 h	8 h	12 h	16 h	24 h	48 h
Mass (g)	46.8 ± 0.8	46.4 ± 0.9	46.7 ± 0.9	47.4 ± 0.8	48.1 ± 0.9	47.0 ± 0.8	46.5 ± 0.9	46.6 ± 0.8	47.9 ± 1.0	47.4 ± 1.0
Diameter (mm)	43.9 ± 0.3	43.9 ± 0.3	43.9 ± 0.3	44.2 ± 0.3	43.9 ± 0.3	43.5 ± 0.3	43.7 ± 0.3	43.5 ± 0.3	43.9 ± 0.3	43.9 ± 0.3

Note. Eggs were placed into hypoxia for 3 d at different time points (0.5–48 h) after oviposition (n = 32–40, N = 355). Data are means ± SE.

success was similar in the 0.5-, 1-, 2-, 4-, 8-, and 12-h treatments compared with control, while none of the eggs in the 16-, 24-, or 48-h treatment groups hatched (fig. 3).

Embryonic Mortality

For eggs from the first night's collection that did not hatch, the stage of embryonic death differed between the treatments that had hatching success typical for green turtles (control, 0.5-, 1-, 2-, 4-, 8-, and 12-h treatments) and the treatments that had low hatching success (16-, 24-, and 48-h treatments; $\chi^2_{CMH} = 6.38$, df = 2, $P < 0.05$). There was proportionally more early stage death in the low hatching success treatments and more middle to late stage death in the high hatching success treatments (fig. 4). The low hatching success from the eggs collected on the second night was caused by the majority of embryos (71%) dying at an early stage of development (Leslie et al.'s [1996] stage 0; no embryo or blood vessels visible).

Discussion

We found that after more than 12 h of exposure to normoxia, the eggs of green turtles did not hatch if they were subsequently exposed to a hypoxic environment. However, after exposure to normoxia for 12 h or less, eggs survived a subsequent 3-d period of hypoxia with no significant decrease in hatching success. Thus, placing eggs into a hypoxic environment within 12 h of oviposition appears to extend preovipositional embryonic arrest. This is further supported by the absence of detectable embryonic development during the 3-d period of hypoxic incubation, while control eggs in normoxia continued to develop. Overall, our results show that preovipositional arrest is

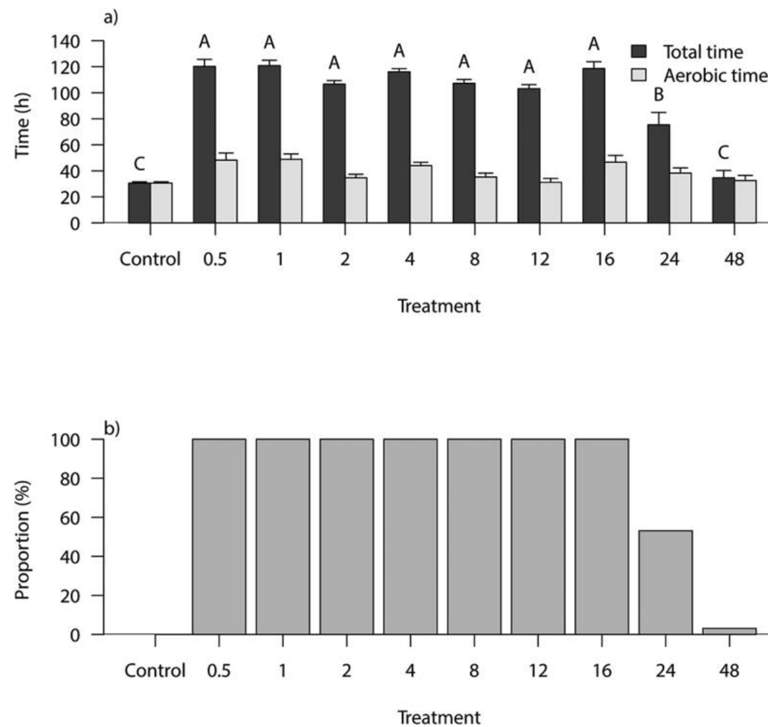

Figure 1. Latency after oviposition until formation of the white spot on eggs (a) and proportion of white spots to form after hypoxia in each treatment group (b). Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Data in a are means ± SE of n = 31–37 per group. In a, dark gray bars represent total time, and light gray bars represent aerobic time (total time excluding time in hypoxia). In b, bars represent the proportion of eggs from each treatment that formed a white spot after being removed from hypoxia. There was no difference between treatment groups in aerobic time (ANOVA; $P > 0.05$). Similar letters indicate that the latency to white spot formation did not differ significantly between the corresponding treatment groups (ANOVA and Tukey's honest significant difference tests; $P < 0.05$).

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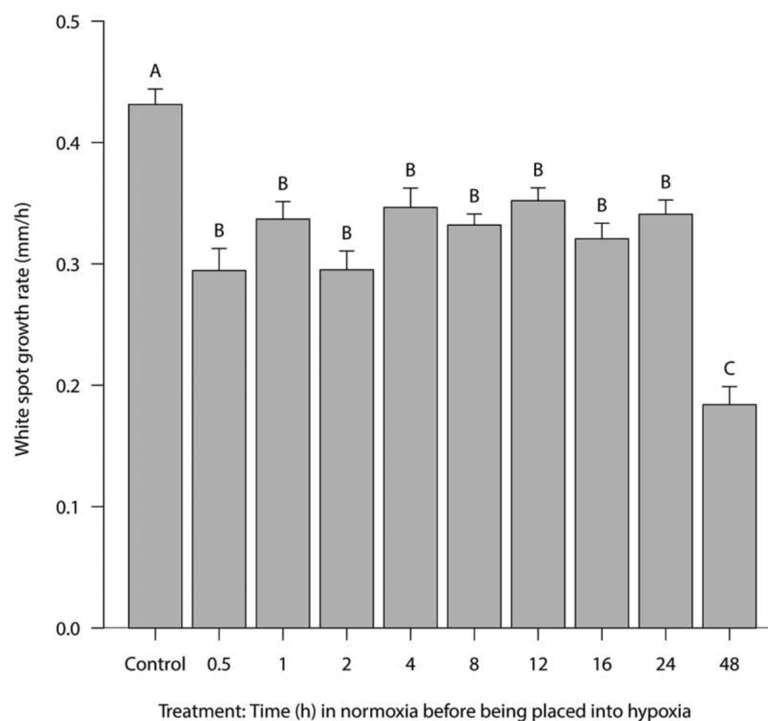

Figure 2. Rate of growth of the white spot on eggs in each treatment group after eggs were removed from hypoxia. Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Data are means ± SE of $n = 25$–37. Similar letters indicate that the white spot growth rate did not differ significantly between the corresponding treatment groups (ANOVA and Tukey's honest significant difference test; $P < 0.05$).

broken within 12–16 h after oviposition. Following this time point, the embryo will not survive further hypoxic episodes. Therefore, we can consider the breaking of embryonic arrest to be an irreversible process. Ecologically, this would mean that for any adverse environmental conditions, such as inundation experienced by the nest after 12–16 h, the embryos would no longer be capable of protecting themselves by pausing oxygen consumption and embryonic development. Our findings build on observations from multiple species of marine and freshwater turtles that the first discernible sign of postovipositional development occurs between 12 and 24 h after oviposition (Miller 1985). Importantly, we found no reduction in hatching success when eggs were placed in an artificial hypoxic incubation environment within 12 h of oviposition. Thus, hypoxic incubation may offer a new tool for conservationists and researchers to increase the time window for safe transportation of marine turtle eggs. We also provide further evidence that the resumption of active embryonic development after preovipositional arrest is initiated by an increase in oxygen availability as eggs transition from the hypoxic oviduct to the normoxic nest (Kennett et al. 1993; Andrews and Mathies 2000; Rafferty and Reina 2012; Rafferty et al. 2013; Rings et al. 2015).

A possible explanation for the higher hatching success (70%–95%) in our hypoxic treatments when compared with those of Rafferty et al. (2013) and Rings et al. (2015) is that our eggs were not chilled and transported a considerable distance during the study. We were able to conduct our experiment close to the nesting site, so long-distance transportation was not necessary. The combination of additional stressors, such as chilling and additional movement, on top of extended retention in a hypoxic environment could potentially explain the increased embryonic mortality found by Rafferty et al. (2013) and Rings et al. (2015).

Table 2: Stage of embryonic development of eggs randomly selected from each treatment

	Control	0.5 h	1 h	2 h	4 h	8 h	12 h	16 h	24 h	48 h
Hypoxia:										
Before	6, 6	6, 6	6, 6	6, 6	6, 6	6, 6	7, 6	7, 7	8, 8	10, 9
After	NA	6, 6	6, 6	6, 6	6, 6	6, 6	6, 7	7, 7	8, 8	9, 9

Note. Embryos were staged according to Miller's (1985) 31-stage developmental chronology. Eggs were placed into hypoxia for 3 d at different time points (0.5–48 h) after oviposition. Eggs ($n = 2$) were opened at the time of collection (control) and before and after each treatment group was placed into hypoxia ($N = 38$). NA, not applicable.

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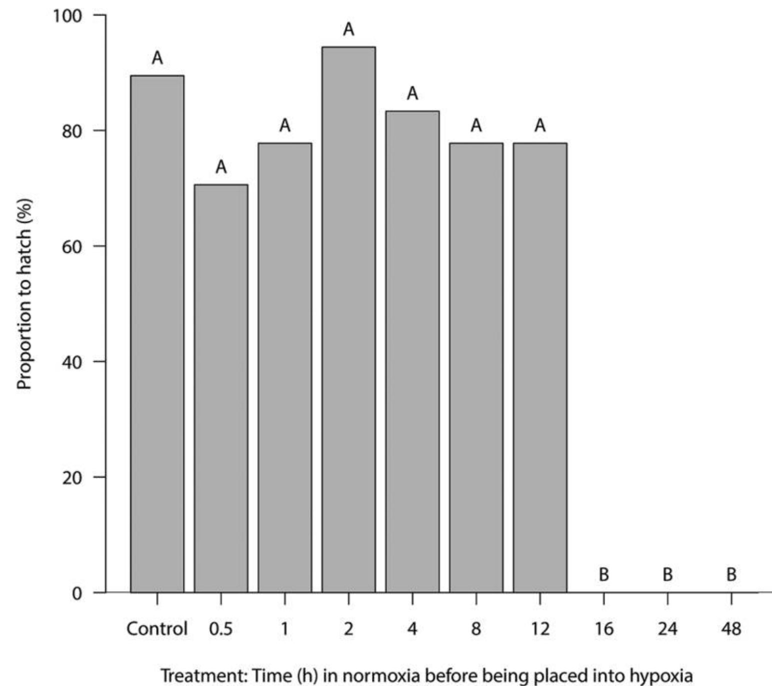

Figure 3. Hatching success (proportion of eggs to hatch) in each treatment where eggs were placed into hypoxia for 3 d after differing amounts of time in normoxia ($n = 16$–20). Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Similar letters indicate that the hatching success did not differ significantly between the corresponding treatment groups (Bonferroni corrected χ^2 test; $P < 0.05$).

However, current research and conservation standards for transportation of turtle eggs over large distances requires chilling the eggs to between 4° and 10°C to slow development and prevent mortality resulting from movement of the eggs (Miller and Limpus 1983). Using the chilling method is not without difficulties, because remote turtle nesting locations are often long distances from the final destination of the eggs. Maintaining eggs at a constant low temperature during transport can be problematic. We know that there have been instances when eggs have become too warm or too cold during transport and have subsequently failed to develop (D. T. Booth, personal communication; C. Cavallo, personal communication; B. Bentley, personal communication). Our new results—combined with the simplicity of our methodology for maintaining eggs in hypoxia—indicate that hypoxia alone may be a viable method for reducing movement-induced mortality during transport.

Movement of the egg after formation of the opaque white spot usually results in the rupture of the membranes that have fused where the white spot has formed (Limpus et al. 1979; Thompson 1985; Deeming 1991). Our results indicate that formation and growth of the white spot is highly dependent on oxygen availability. When eggs were placed into hypoxia, the formation of a white spot was delayed, and white spots that had already formed did not grow for the duration of the hypoxic incubation period. Furthermore, our results suggest that formation and subsequent growth of the white spot is not necessarily a sign that the embryo is successfully developing, unlike what has been suggested previously for turtles (Thompson 1985; Deeming and Thompson 1991; Beggs et al. 2000; Booth 2000) and crocodiles (Webb et al. 1983*a*, 1983*b*). We found that eggs that were placed into hypoxia after arrest had broken (>12 h from oviposition) still formed white spots that continued to grow once the eggs were returned to a normoxic environment, but the majority of embryos from these treatments died at an early stage of development. This suggests that the white spot formed and grew, despite the absence of an actively developing embryo. A similar phenomenon has been reported in lizard eggs, where dead eggs continued to take up water during the first half of incubation (Warner et al. 2011). Perhaps the formation and growth of the white spot is influenced by other passive environmental factors, such as the drying of the shell and hydration of the albumin when the egg is placed into the nest, which subsequently causes the vitelline membrane to migrate to the top pole of the egg and adhere (chalk; Thompson 1985; Webb et al. 1987*b*). Alternatively, the development of embryos that are exposed to hypoxia after 12 h of normoxia may gradually slow until it stops completely, but the white spot may grow during the period before the embryo dies.

Somewhat surprisingly, in the case of eggs exposed to hypoxia before the breaking of arrest (≤12 h from oviposition), there was still a noticeable reduction in the rate of white spot growth once the eggs were returned to normoxia, even though the eggs in these treatments had a similar hatching success to those from the control treatment. Although this did not result in increased embryonic mortality, it suggests that extended retention of eggs in the oviduct by the mother—and hence

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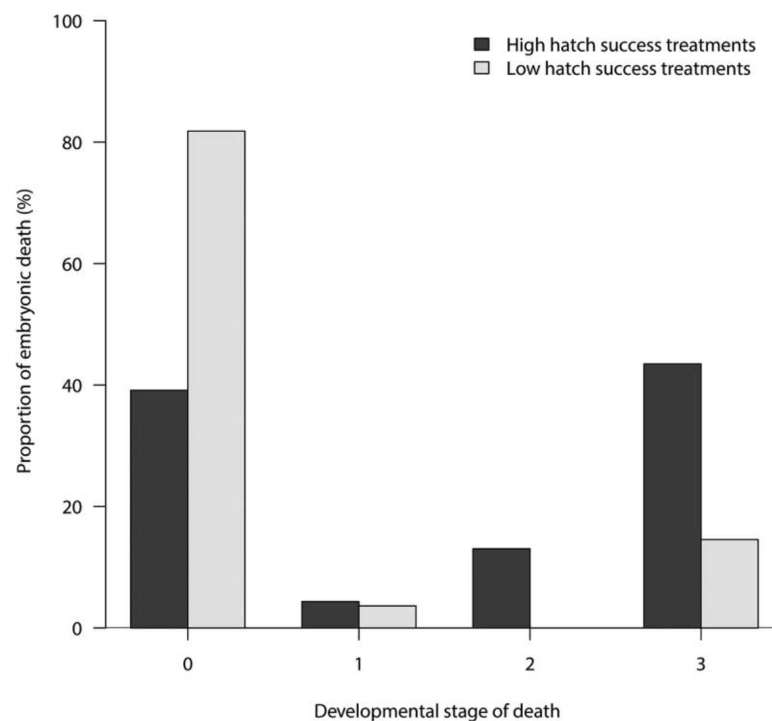

Figure 4. Proportion of embryos from the first collection night that died at each developmental stage. Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Dark gray bars indicate treatments with high hatching success (control and 0.5–12-h treatments; $n = 23$), and light gray bars indicate treatments with low hatching success (16–48-h treatments; $n = 55$). Embryos were staged according to Leslie et al.'s (1996) field-staging method, where embryos are classified into four stages of development aging from 0 to 3.

extended preovipositional arrest—may compromise early development, as has been documented in a leatherback turtle population (Rafferty et al. 2011). Ecologically, this means that turtles may be limited in their ability to utilize embryonic arrest as a strategy to avoid adverse nesting conditions.

Selection for extended retention and development of eggs in the oviduct has been suggested as the key requirement for the evolution of viviparity (Shine 1985; Shine and Guillette 1988). It has been proposed that for this to occur, selection for traits that increase in utero availability of oxygen for the embryo must simultaneously occur (Andrews 2002; Parker and Andrews 2006; Parker et al. 2010). It has been shown that the hypoxic oviducal environment in turtles prevents further development in utero and hence may constrain the evolution of viviparity (Rafferty et al. 2013). Critically, increased development of the embryo within the mother would require the breaking of preovipositional embryonic arrest. This could then result in the embryonic and egg shell membranes fusing while the egg is still within the mother. Consequently, embryos would then be susceptible to mortality either from exaggerated movements of the mother while they are still within the oviduct or from the movement they experience when they are eventually laid into the nest. Thus, in the context of the evolution of viviparity, selection for even a slight increase in development of the embryo within the mother would be deleterious.

There is mixed evidence from squamates that suggests that either they are not susceptible to movement-induced mortality (Marcellini and Davis 1982) or they have overcome this risk by delaying fusion of the membranes until after oviposition (Aubret et al. 2015). The roadblock to the evolution of viviparity—imposed by the nature of hypoxic embryonic arrest—could be overcome only through a dramatic change in the embryonic biology of turtles. Changes that prevent fusion of membranes that causes susceptibility to movement-induced mortality, increase the oxygen availability, and allow retention of eggs in the oviduct would be required to occur simultaneously. Therefore, fusion of embryonic membranes and subsequent susceptibility of early stage embryos to movement-induced mortality likely represents a further constraint on the evolution of viviparity within turtles. This increases our understanding of why these almost exclusively aquatic animals are dependent on a return to the terrestrial environment to maintain their oviparous life-history strategy.

Turtle life-history patterns are influenced by their ability to control reproductive timing. In times of poor environmental conditions, when nesting is delayed, the ability of embryos to remain arrested is paramount because movement would kill any embryo if it continued to develop within the mother. This highlights that arresting development is critical for maintaining reproductive success of this taxon. Further investigation of this physiological mechanism in other taxa (such as chameleons) that likely display preovipositional embryonic arrest is warranted (Rafferty and Reina 2012). In turtles, a physiological constraint of low oxygen in the oviduct is utilized as an ad-

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aptation for greater control over reproductive timing, influencing species life history and therefore ecology and evolution. Future research should address whether hypoxic oviducts are the ancestral state for turtles and all egg-laying taxon or if they have evolved this trait as a result of selection pressure to permit greater flexibility in their reproductive timing and, in turn, improve their ecological success. Investigation of the oxygen availability in the oviducts of other extant egg-laying taxa is warranted.

The difference we found between the hatching success of eggs collected on the first versus the second collection nights could potentially be explained by the difference in developmental timing when the eggs were moved from the laboratory back to the nesting beach for burial in the artificial nests. Embryos from the first collection would have been between 14 and 11 d of development, depending on whether they were placed into hypoxia for 3 d or not. However, embryos from the second collection would have been between 9 and 6 d of development when they were moved. Because these embryos were younger, they may have been more susceptible to mortality from even the slightest movement (Limpus et al. 1979; Parmenter 1980; Miller and Limpus 1983). Despite us carefully handling the eggs and minimizing movement during egg burial, it appears that their sensitivity to movement was too great. Our embryonic staging data also support this hypothesis, because the majority of embryos died at an early stage of development. Eggs from each treatment were randomly dispersed to different depths within the reburied nests, so any random effect of egg mortality on neighboring eggs of different treatments would have been controlled for.

In conclusion, our findings provide the first experimental evidence that preovipositional embryonic arrest is broken between 12 and 16 h after laying in green turtle eggs. We have also shown that it is possible to place eggs back into hypoxia before this time has elapsed in order to extend preovipositional arrest and delay development, with no negative impact on hatching success. However, developmental arrest is broken at a time point between 12 and 16 h after oviposition. Consequently, embryos die if they are then incubated in hypoxia for a substantial period. This information provides further evidence that eggs should be relocated or moved only under normoxic conditions within 12 h of oviposition. Additionally, our findings provide the first empirical evidence for the potential to use hypoxia—instituted within 12 h of oviposition—to extend developmental arrest and allow safe transportation of turtle eggs without risk of movement-induced mortality or the need for chilling. Thus, our findings not only add to our basic physiological understanding of how preovipositional embryonic arrest functions but also provide a potential new tool for researchers and conservation managers who work with the egg life-history stage of turtles. Our findings also have important implications for our understanding of the selection pressures that have constrained the evolution of reproductive biology in turtles. Preovipositional embryonic arrest provides protection from movement-induced mortality. However, this adaptation also represents a roadblock to evolution of viviparity, since even slight increases in the stage of embryonic development before oviposition would, by necessity, result in the fusion of embryonic membranes and thus render the embryo susceptible to movement-induced mortality. It seems likely that these factors have provided strong selection pressure for oviducal hypoxia in gravid turtles.

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