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The Impact of Extended Preovipositional Arrest on Embryonic Development and Hatchling Fitness in the Flatback Sea Turtle*

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ABSTRACT

Turtle embryos pause development before oviposition in a process known as preovipositional arrest. Embryonic development arrests due to hypoxia (low oxygen) in the maternal oviducts and resumes only after exposure to normoxia when eggs are laid. Recently, several studies have hypothesized that the prolonged periods of preovipositional arrest may have a detrimental effect on embryo survival and development after eggs are laid. We tested this hypothesis by comparing embryo survival (determined by white spot formation and hatching success) and hatchling fitness (measured by self-righting, crawling, and swimming ability) of flatback sea turtle (*Natator depressus*) eggs following incubation in hypoxic (~1%) and normoxic (~21%) treatments for 5 d immediately following oviposition. We also measured embryo survival and hatchling fitness when eggs were incubated in hyperoxic conditions (42% oxygen), to determine whether hyperoxia could improve developmental outcome or whether some consequence of oxidative stress might manifest. Eggs incubated in hypoxia remained arrested during the 5-d treatment, and 97.5% of the eggs successfully recommenced development after exposure to normoxia when the treatment finished. At treatment commencement, 100% and 97.5% of eggs in the hyperoxic and normoxic treatments, respectively, began developing. Although hatching success was significantly lower following hypoxia (15%) compared to normoxia (80%) and hyperoxia (85%), hatchings from the hypoxic treatment were larger (carapace length and width and plastron length) than normoxic hatchlings. Similarly, hypoxic hatchings also swam significantly faster than hyperoxic hatchlings. Considering larger hatchlings may have a greater chance of survival, the production of larger hatchings may offset the high cost (lower hatching success) when preovipositional arrest is prolonged. Hyperoxia does not appear to have deleterious consequences for development.

Introduction

Sea turtles are obligate egg layers that do not provide parental care of nests or neonates, so their developmental success or failure is highly dependent on the maternal investment in the components of eggs and the environment in which the eggs incubate (Ackerman 1997; Wallace et al. 2006, 2007). Female turtles migrate to nesting grounds after having acquired sufficient energetic resources to travel long distances and to produce large clutches of energetically expensive eggs, with a frequency that is heavily influenced by resource availability and distribution (Reina et al. 2002, 2009; Saba et al. 2007). Mating generally occurs near the nesting site, after which females produce and oviposit sequential clutches of eggs that develop and hatch without further influence from the mother, having already been provisioned with internal resources for embryonic growth (Wallace et al. 2006) and the first few days of life of hatchlings (Jones et al. 2007). Development of the embryo commences before oviposition, with the remainder occurring while in the nest environment on the nesting beach. Approximately 6 d after ovulation, the embryo inside each fully formed sea turtle egg downregulates growth and development in a process known as preovipositional arrest (Rafferty and Reina 2012). Embryos enter into this state when they become gastrulas and remain arrested while retained inside the oviduct (Ewert 1985; Miller 1985). The duration of maternal egg retention between gastrulation and oviposition therefore dictates how long embryos remain in a state of preovipositional arrest. The duration of egg retention varies widely among turtle species and can range from 5 or 6 d in the leatherback turtle (*Dermochelys coriacea*) to 63 d in the olive ridley turtle (*Lepidochelys olivacea*; Miller 1997; Plotkin et al. 1997). Some species of freshwater turtle (*Deirochelys reticularia*) are even capable of retaining eggs between seasons (Buhlmann et al. 1995). An extremely low-oxygen (hypoxic) environment in the oviducts of gravid female turtles is known to maintain embryonic preovipositional arrest in utero. Active embryonic development only recommences following exposure to atmospheric oxygen levels after eggs are laid (Rafferty

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et al. 2013). There is an ongoing influence of the environment on the subsequent completion of development of embryos, as well as on the fitness of emerged hatchlings (Lee and Hays 2004), through factors such as temperature (Booth 2006; Rafferty and Reina 2014), concentrations of respiratory gases (Wallace et al. 2004), egg location within the nest (Ralph et al. 2005), and sand moisture (Wallace et al. 2004).

Embryonic diapause is an important strategy for regulating the timing of development and egg laying or birth in several vertebrate taxa (Renfree and Shaw 2000; Lopes et al. 2004; Rafferty and Reina 2012). For oviparous reptiles, there are numerous possible evolutionary advantages associated with preovipositional arrest that include (1) halting embryonic development so that turtle mothers have the flexibility to select the most suitable conditions to lay their eggs, (2) ensuring that all eggs are at the same developmental stage when laid so that synchronous development and hatching can occur, and (3) preventing movement-induced mortality of embryos during laying, which arises at later stages of development if the sensitive connection between the vitelline and shell membranes becomes mechanically damaged during oviposition (Ewert 1979; Andrews 2004; Rafferty and Reina 2012).

Despite these evolutionary advantages, emerging evidence suggests that the duration of time embryos spend arrested in utero may have a detrimental effect on embryonic growth and survival following oviposition. Bell et al. (2004) showed that unhatched leatherback turtle embryos predominantly died as arrested gastrulas, caused by failing to recommence development postoviposition. Subsequent research demonstrated an increasing likelihood of failure to recommence development after oviposition in this population with greater duration of preovipositional arrest (Rafferty et al. 2011). Early embryonic death is a serious problem for this species of critically endangered sea turtle, which has an extremely low average global hatching success of approximately 50% (Bell et al. 2004). Extended periods of preovipositional arrest have also been shown to increase mortality during the initial stages of development, following oviposition, in species of both freshwater turtles (western oblong turtle *Chelodina oblonga*) and sea turtles (green turtles *Chelonia mydas*; Rafferty et al. 2013). If extended periods of hypoxia in utero can result in a failure of embryos to recommence development after oviposition, it is possible that a remedial management strategy may be to expose nests of turtles to elevated concentrations of oxygen shortly after egg laying is completed. Such hyperoxic conditions may provide extra stimulus for embryos to begin development that would not occur otherwise, detectable as increased developmental success. This approach to improve developmental outcome has never been reported, but it seems worthy of investigation. However, hyperoxia can result in the increased production of reactive oxygen species, and these free radicals may cause oxidative stress to developing organisms (Gille and Sigler 1995). While there appears to be capacity for animals to ameliorate this stress via antioxidant systems (Gille and Sigler 1995; Lushchak et al. 2005; Lushchak 2011), the potential for hyperoxia to cause oxidative stress on developing reptile embryos has never been reported and needs investigation before hyperoxia can be considered as a possible remedial action to address poor developmental success.

Therefore, the primary aim of this study was to investigate whether simulating extended periods of preovipositional developmental arrest, by keeping eggs in a hypoxic environment, had a significant detrimental effect on survival and development of flatback sea turtle embryos after oviposition and hatchling fitness (e.g., self-righting, crawling, and swimming ability) after hatching. Further, we investigated whether exposure to increased oxygen levels had a detectable positive or negative influence on early embryonic development and hatchling fitness.

Material and Methods

Egg Collection

Two flatback sea turtle clutches consisting of 120 eggs (53 from clutch 1 and 67 from clutch 2) were collected during oviposition in June 2013 from Bare Sand Island (12°32.4′S, 130°25.0′E), located approximately 50 km west of Darwin, Northern Territory, Australia (fig. 1). Eggs were collected from nesting females using a plastic ziplock bag placed directly below the cloaca and evenly divided and distributed between three experimental treatments within ~10 min of collection (table 1).

Experimental Design

To assess the influence that extended periods of preovipositional arrest had on embryonic development, we incubated eggs in three experimental treatments consisting of hypoxic, hyperoxic, and normoxic (control) environments for 5 d following oviposition (fig. 2). Hypoxia is known to prolong preovipositional arrest, allowing us to compare embryonic development in this treatment to the hyperoxic and normoxic treatments (Rafferty et al. 2013). Five or six days is about the longest period that can elapse between the expected oviposition date and the actual oviposition date in natural populations of leatherback turtles (Reina et al. 2002; Rafferty et al. 2011), so we used it to represent a period of extended preovipositional arrest.

Experimental treatments were created using airtight Perspex containers measuring 26 cm × 15 cm × 11 cm (Resi-Plex Plastics, North Geelong, Australia). Hypoxic and hyperoxic environments were generated by pumping pure nitrogen gas or 42% oxygen-nitrogen blend (Air Liquide, Melbourne), respectively, into each container at a flow rate of 9 L/min. The normoxic treatment involved exposure of eggs to normal atmospheric oxygen concentrations. Hypoxic and hyperoxic gases were humidified by bubbling them through distilled water in an airtight chamber before pumping them into the airtight containers via an inflow valve. An outflow valve at the opposite end of each container allowed gases to escape. The oxygen tension of the gas leaving the container was recorded using an oxygen sensor (Analytical Industries, Po-

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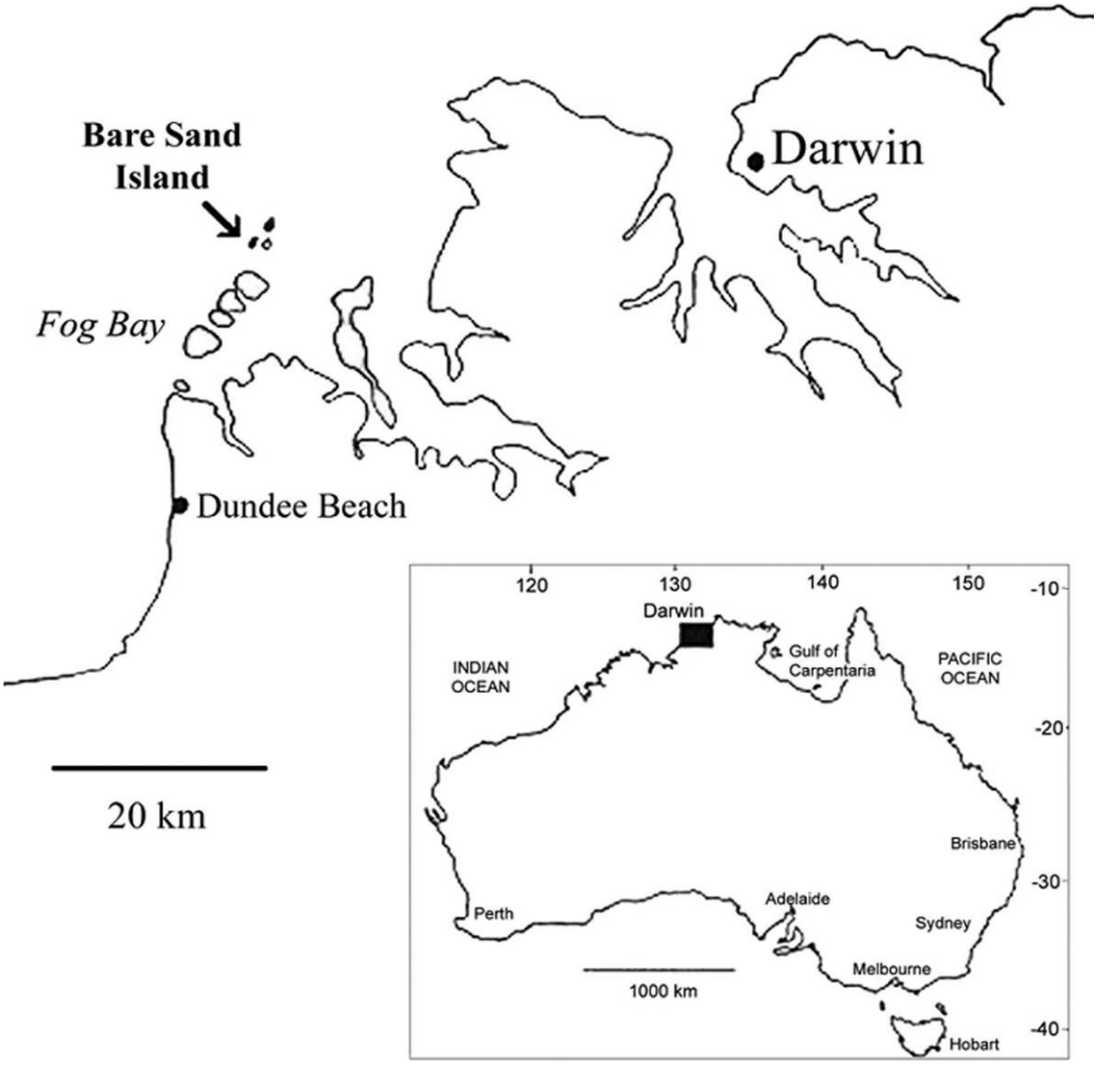

Figure 1. Bare Sand Island, Fog Bay, ~50 km west of Darwin, Northern Territory. *Inset*, location of Darwin, Australia. (Map courtesy of http://www.austurtle.org.au.)

mona, CA) and a data collection device (Pasco, Roseville, CA). Gases were pumped continuously through the relevant container as eggs were being placed inside. Approximately 3 min after the last egg was placed in a container, the valves were closed and the box was sealed, ensuring that the desired oxygen concentration for that treatment had been achieved. Normoxic treatments were exposed to atmospheric oxygen by leaving the container lid open, until it was closed for incubation. Within each container, eggs were placed on a thin wire mesh to avoid contact with moisture from gas humidification.

Each sealed, airtight container was then placed in an insulated container with chilled sand (~5°C) and cold gel packs,

Table 1: Allocation of eggs according to treatment and clutch

	Hypoxia (1% oxygen)	Normoxia (21% oxygen)	Hyperoxia (42% oxygen)
Clutch 1	19	14	20
Clutch 2	21	26	20
Total eggs per treatment	40	40	40

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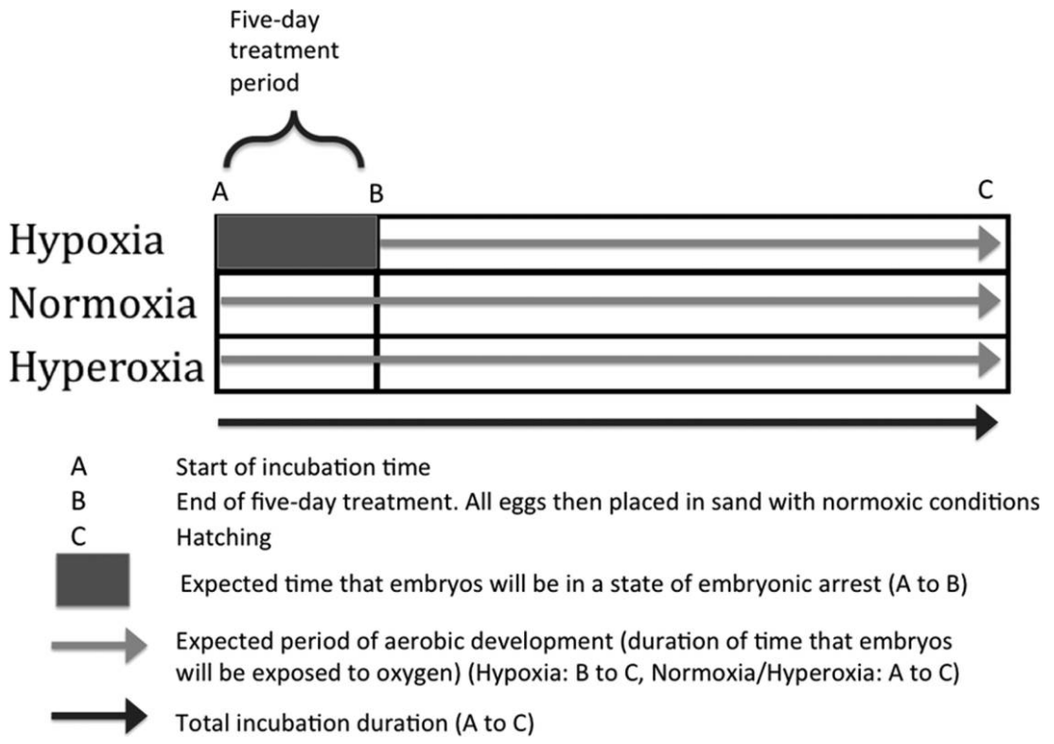

Figure 2. Experimental schedule. A color version of this figure is available online.

which maintained an average temperature <10°C, in order to halt embryonic development during transport from the island (Harry and Limpus 1989). Eggs remained chilled during air and road transport to Charles Darwin University, Darwin, which did not exceed 14 h from the time of egg collection. On arrival, each treatment was exposed to the relevant gas treatment once more for 3 min (using the method outlined above) before being placed in separate GQF HovaBator incubators (model 1632; Grandview Management, Baldivis, Australia) set at 30°C. Exposure to the relevant gas continued in each treatment for 3 min each day during the 5-d experimental period, after which all treatments ceased. Oxygen concentration remained stable once containers were sealed shut. Eggs were then exposed to atmospheric oxygen and placed in sand (~7% moisture content by mass) to continue development until hatching.

Egg Incubation

It was assumed that preovipositional arrest was maintained and that no development occurred while eggs were chilled (Harry and Limpus 1989). Therefore, treatment commencement began on placement of eggs in incubators. Incubation duration was calculated in days from treatment commencement to full emergence of the hatchlings from their shell (Miller et al. 2003). Given that hypoxia was hypothesized to maintain preovipositional arrest and simulate extended egg retention in the mother's oviduct during the 5-d experimental period (Rafferty et al. 2013), we defined aerobic developmental time to be a measure of active development calculated from the first day eggs were exposed to at least 21% oxygen (normoxia) until hatching (fig. 2). Thus, for the hypoxic treatment, aerobic development began at treatment cessation on exposure to normoxia 5 d after the treatment had first commenced (fig. 2).

During the initial stages of incubation, eggs were visually checked daily (without removing them from the incubator) for the formation of a white spot, which we considered to represent the breaking of preovipositional arrest and active recommencement of development. Eggs that had not formed a white spot after 8 d of exposure to aerobic conditions were candled to check for signs of development (fig. 3). Candling is a technique whereby a light source is shone into the egg to illuminate the internal egg environment and allow visual monitoring of embryo development. Eggs displaying no signs of development after 8 d were assumed to be dead and were taken out of the incubators. The mean number of days that eggs took to form white spots (on exposure to normoxia or hyperoxia) was calculated for each treatment. For the remainder of incubation, eggs were routinely monitored for development. Dead eggs were removed from incubators and the developmental stage at death was determined using Miller's (1985) 31-stage classification for sea turtle embryonic development.

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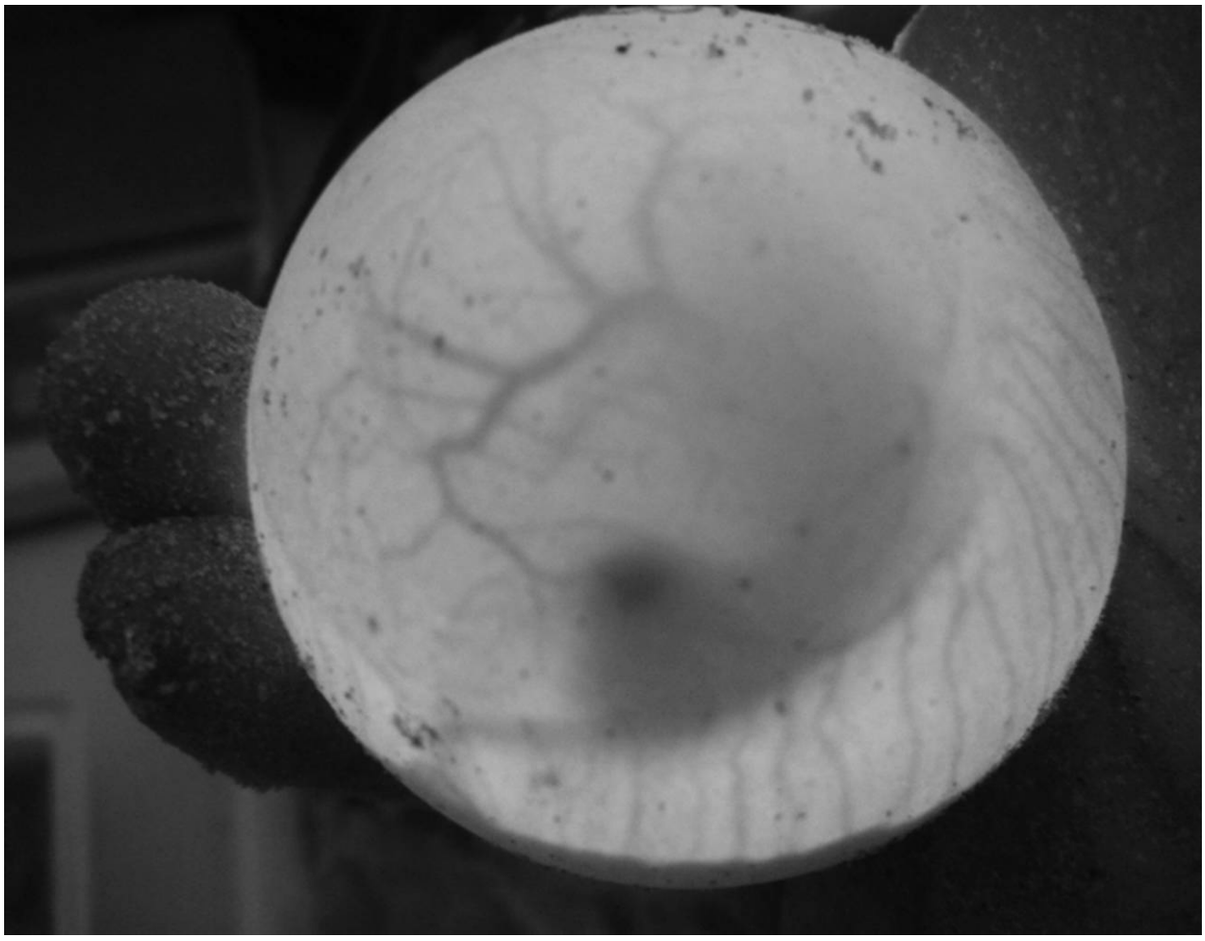

Figure 3. Egg candling showing embryonic development inside the egg. A color version of this figure is available online.

Hatching Success and Hatchling Fitness

Hatching success was determined for each treatment as the proportion of all eggs to hatch from those originally included in each treatment. After hatching, hatchlings were placed in incubators at 28°C in individual compartments with moist sand, where they were kept for 48 h to absorb their yolk. Hatchling mass was then recorded (g), and the following morphometric measurements were taken using digital calipers (mm): straight carapace length and width (at the widest point), left and right flipper length, plastron length and width, and head width.

The self-righting ability of hatchlings was assessed using methods previously described (Read et al. 2012). Given that body temperature affects hatchling righting response (Steyermark and Spotila 2001*a*), hatchlings were kept in 27°C incubators for a minimum of 15 min before testing. Hatchling body temperature was recorded before testing (using a ProExotic tempgun PE2) and was between 26° and 27°C. Testing involved placing hatchings on their carapace in containers (40 cm × 30 cm) containing moist sand and recording the time taken to turn themselves onto their plastron. Each hatchling was allowed one practice trial before undergoing serial testing until three successful self-righting events took place, or a maximum of six attempts had occurred. If hatchlings were unable to self-right within a 5-min time period, the trial was abandoned and they were turned onto their plastron (and given a 5-min rest before the next trial). The mean self-righting time was recorded (from successful self-righting attempts). A propensity score between 0 and 6 was also given to each hatchling based on their ability to self-right (table 2). Lag time (the time taken before the hatchling begins its attempt to self-right) and mechanical righting time (the actual time taken to turn over) were both measured (Steyermark and Spotila 2001*a*). In every trial the lag time was 0 s, so self-righting time was equivalent to mechanical righting time.

Crawling ability was also measured as previously described (Ischer et al. 2009; Read et al. 2012) using PVC guttering pipe (300 cm length × 15 cm width) lined with moist sand. Swimming ability was measured using a PVC guttering pipe (510 cm length × 15 cm width), sealed at both ends and containing saltwater (26°C). Both crawling and swimming trials were set up in darkness with a torch positioned at one end to stimulate unidirectional movement toward the light

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Table 2: Righting propensity scoring for self-righting trials based on previous studies (Booth et al. 2013)

Score	Condition
0	No righting event in six trials
1	One righting in six trials
2	Two rightings in six trials
3	Three rightings in six trials
4	Three rightings in five trials
5	Three rightings in four trials
6	Three rightings in three trials

source. Hatchlings were timed from beginning movement at one end of the pipe to reaching the opposite end. Each hatchling was given one practice trial before undergoing three additional trials. Mean crawling and swimming speeds (cm/s) were calculated for each hatchling.

Data Analysis

Statistical analyses were performed using R software (R Core Team 2013). Initially, Shaprio-Wilks and Fligner-Killeen tests were used to assess normality and equal variance. Two-factor ANOVA (with treatment and clutch identity as factors) and Tukey's HSD tests were then used to compare average time to white spot formation, total incubation duration, hatchling morphology, swimming speed, and self-righting propensity score between treatments and clutches. Self-righting time and crawling speed data were not normally distributed ($P < 0.01$) and had unequal variance, respectively, so nonparametric Kruskal-Wallis and post hoc Mann-Whitney-Wilcoxon tests were used to identify difference between treatments and clutches. Hatchlings that did not self-right during any of the six attempts were excluded from analysis. Statistical significance was assumed if $P < 0.05$, and all values reported are mean ± standard error.

Results

Egg Development and White Spot Formation

Development occurred normally after treatment commencement in the hyperoxic and normoxic treatments, with white spot formation occurring in 100% and 97.5% of the eggs, respectively. Eggs in the hypoxic treatment remained in preovipositional arrest and did not develop white spots during the treatment period. On exposure to normoxic conditions after treatment cessation (from day 5 onward), 97.5% of the eggs in the hypoxic treatment successfully developed a white spot (table 3). There was no significant difference in the number of days to white spot formation after exposure to oxygen between treatments ($\chi^2 = 0.01$, df = 2, $F = 0.02$, $P = 0.98$) or clutches ($\chi^2 = 0.87$, df = 1, $F = 2.09$, $P = 0.15$; table 3).

Although mean total incubation time was significantly longer in the hypoxic treatment compared to the hyperoxic and normoxic treatments ($\chi^2 = 88.26$, df = 2, $F = 41.68$, $P < 0.001$; table 3), no significant differences were observed in aerobic development time. On the other hand, both incubation and aerobic development time were significantly longer in the hyperoxic treatment compared to the normoxic treatment ($\chi^2 = 88.28$, df = 2, $F = 38.79$, $P = 0.02$ and $\chi^2 = 9.51$, df = 2, $F = 4.5$, $P = 0.02$, respectively; table 3). Clutch identity had no significant effect on total incubation or aerobic developmental time.

Embryonic Death

A total of 34, 8, and 6 embryos died during development in the hypoxic, normoxic, and hyperoxic treatments, respectively, with approximately equal numbers from each clutch of eggs. Of the eggs that died in the hypoxic treatment, the majority (27 of 34) died at early developmental stages. There was no apparent trend in mortality in the normoxic or hyperoxic treatments, probably because most eggs successfully developed to hatching. However, of the unsuccessful eggs in the normoxic and hyperoxic treatments, over half died during the final stages of embryonic development (table 4).

Hatchling Morphology

Treatment had a significant effect on carapace length ($\chi^2 = 56.12$, df = 2, $F = 10.26$, $P < 0.001$) and width ($\chi^2 = 70.35$, df = 2, $F = 7.94$, $P < 0.001$), plastron length ($\chi^2 = 77.55$, df = 2, $F = 8.43$, $P < 0.001$), and mass ($\chi^2 = 17.57$, $F = 388$, df = 2, $P < 0.05$), with all variables being significantly greater in both the hypoxic and hyperoxic treatments than the normoxic treatment (table 5). Treatment had no effect on flipper length, head, or plastron width.

There was also a significant difference in carapace length ($\chi^2 = 102.30$, df = 1, $F = 37.40$, $P < 0.001$) and width ($\chi^2 =$

Table 3: Developmental schedule of *Natator depressus* eggs during incubation ($n = 40$ eggs per treatment)

	Hypoxia (1% oxygen)	Normoxia (21% oxygen)	Hyperoxia (42% oxygen)
White spot development during 5-d treatment (%)	0	97.50	100
White spot and chalking after exposure to normoxia (%)	97.50	97.50	100
No. eggs to hatch (%)	6 (15)	32 (80)	34 (85)
Mean total incubation duration (d)	58.00 ± .52	52.09 ± .24	53.15 ± .29
Mean aerobic developmental time (d)	53.00 ± .52	52.09 ± .24	53.15 ± .29
Mean no. days to white spot formation after exposure to normoxia (d)	2.69 ± .20	2.67 ± .08	2.68 ± .08

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Table 4: Embryonic mortality staging of *Natator depressus* eggs according to treatment

Stages of embryonic mortality[a]	Hypoxia (1% oxygen)	Normoxia (21% oxygen)	Hyperoxia (42% oxygen)
Early embryonic death (Miller's stage 0–16; %)	79.40	25	20
Mid embryonic death (Miller's stage 17–23; %)	8.80	25	20
Late embryonic death (Miller's stage 24–31; %)	11.80	50	60
Total number of dead eggs (*n*)	34	8	6

[a]Early-, mid-, and late-staging groupings are based on those previously reported (Leslie et al. 1996; Rafferty et al. 2011).

47.34, df = 1, $F = 10.69$, $P < 0.01$), flipper length ($\chi^2 = 78.70$, df = 1, $F = 19.11$, $P < 0.001$), plastron length ($\chi^2 = 56.45$, df = 1, $F = 12.27$, $P < 0.001$), and mass ($\chi^2 = 161.85$, df = 1, $F = 71.45$, $P < 0.001$) between clutches (table 6). However, clutch had no effect on head or plastron width (table 6).

Self-Righting Ability

Two hatchlings were excluded from statistical analysis because they failed to self-right during the six attempts (both were from the same clutch, one from the normoxic treatment and one from the hyperoxic treatment). All other hatchlings attempted to self-right immediately on being placed on their back (i.e., lag time was 0 s in every trial). Neither treatment nor clutch had a significant effect on mean time to self-righting or righting propensity (tables 5, 6).

Crawling and Swimming Ability

There were significant differences in crawling and swimming ability between treatments ($H = 9.41$, df = 2, $P < 0.01$ and $\chi^2 = 120.46$, df = 2, $F = 13.21$, $P < 0.001$, respectively; table 5). Although hatchlings in the hyperoxic treatment crawled faster than those in the normoxic treatment ($W = 665$, $P < 0.01$), they swam significantly slower than those in both the normoxic ($P < 0.01$) and hypoxic ($P < 0.01$) treatments. No significant differences in crawling or swimming ability existed between normoxic and hypoxic treatments or crawling ability between the hypoxic and hyperoxic treatments. However, hatchlings in the hyperoxic treatment swam significantly faster than those in the hypoxic treatment ($P < 0.01$; table 5). Although hatchling crawling speed was significantly different between clutches ($H = 8.94$, df = 1, $P < 0.01$), swimming speed did not differ (table 6).

Discussion

Our results show that preovipositional arrest is prolonged in flatback turtle embryos in a hypoxic environment. This is consistent with studies on other species of freshwater (*Chelodina oblonga, Chelodina longicollis, Emydura macquarii*) and sea (*Chelonia mydas*) turtles whose eggs also remain arrested on exposure to hypoxia after oviposition (Rafferty et al. 2013). Furthermore, this is the first study to investigate the effect that prolonged preovipositional arrest has on hatching success and hatchling size and fitness, the latter of which can have significant implications for hatchling survival in the wild, especially given the intense predation that hatchlings face on emergence from the nest (Miller et al. 2003). Our results show that an extended period of preovipositional arrest may potentially decrease hatching success but may positively influence hatchling size and fitness. The majority of unsuccessful eggs in the hypoxic treatment died early during embryonic development, consistent with previous studies on sea turtles that have also reported an increase in embryonic death during this period due to extended egg retention (Bell et al. 2004; Rafferty et al. 2013). Extended egg retention may occur as a result of false crawls, disturbance on the nesting beach (e.g., humans/artificial lights), or weather conditions that prevent females from nesting for a time. All species of sea turtles display a range of interesting periods, with nesting generally occurring with a range of 5 d or more between earliest and latest date of oviposition (Miller 1997; Reina et al. 2002).

The initial delayed development of white spots in the hypoxic treatment is presumably a result of delayed development, illustrating that preovipositional developmental arrest was extended in this treatment. In turtle embryonic development, the mechanism by which oxygen controls the process of preovipositional arrest remains unknown, and future research should endeavor to identify whether oxygen concentration is a trigger for gene expression or whether cells are simply starved of oxygen while in the hypoxic environment in the oviduct. Our study found that extended egg retention did not influence the time taken for embryonic development to recommence, but it appeared to interfere with the early stages of development (Miller's stage 16 and below) because higher numbers of eggs died during this time than in the normoxic control treatment.

The mean time to white spot formation on exposure to oxygen was consistent across all treatments. Similar findings are reported for the Australian northern long-necked turtle (*Chelodina rugosa*), whose eggs remain in a prolonged state of preovipositional arrest after they are laid into underwater nests (Kennett et al. 1993). However, our findings contrast with those on several other freshwater (*C. oblonga, C. longicollis, E. macquarii*) and sea (*C. mydas*) turtle species, where time to white spot formation was significantly longer with increased duration (3, 6, or 9 d) in hypoxia (Rafferty et al. 2013). This difference between species in their response to extended

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Table 5: Mean *Natator depressus* hatchling morphometric and hatchling fitness measurements (±SE) according to treatment

	Hypoxia (1% oxygen)	Normoxia (21% oxygen)	Hyperoxia (42% oxygen)	*P*	Significant pairwise comparisons
No. hatchlings (*n*)	5	29	32		
Morphometric measurement:					
Carapace length (mm)	58.10 ± .43^{A}	55.84 ± .40^{B}	57.62 ± .38^{C}	<.001*	AB .02, BC <.01
Carapace width (mm)	49.24 ± .56^{A}	46.32 ± .49^{B}	48.18 ± .35^{C}	<.001*	AB .02, BC <.01
Flipper length (mm)	44.17 ± .68	43.62 ± .46	43.60 ± .39	.84	
Head width (mm)	15.63 ± .15	15.20 ± .25	15.32 ± .07	.64	
Plastron length (mm)	48.06 ± .56^{A}	44.41 ± .49^{B}	46.07 ± .37^{C}	<.001*	AB <.01, BC .01
Plastron width (mm)	39.00 ± .55	37.98 ± .44	39.12 ± .36	.11	
Mass (g)	32.28 ± .81^{A}	32.13 ± .51^{B}	33.18 ± .31^{C}	<.05*	BC .02
Hatchling fitness:					
Self-righting time (s)	15 ± 4.62	13.86 ± 1.71 ($n = 28$)	22.03 ± 4.14 ($n = 31$)	.93	
Righting propensity	6.00 ± .00	5.66 ± .22	5.72 ± .20	.76	
Crawl speed (cm/s)	7.21 ± .45^{A}	7.20 ± .30^{B}	9.10 ± .48^{C}	<.01*	BC <.01
Swim speed (cm/s)	16.59 ± .75^{A}	15.69 ± .34^{B}	13.16 ± .42^{C}	<.001*	AC <.01, BC <.01

Note. Superscript letters indicate significant differences between treatment groups.
*$P < 0.05$.

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Table 6: Mean *Natator depressus* hatchling morphometric and hatchling fitness measurements (±SE) according to clutch

	Clutch 1	Clutch 2	*P*
No. hatchlings (*n*)	34	32	
Morphometric measurement:			
Carapace length (mm)	57.17 ± .35	55.45 ± .27	<.01*
Carapace width (mm)	48.46 ± .41	46.36 ± .42	<.01*
Flipper length (mm)	44.71 ± .33	42.54 ± .37	<.01*
Head width (mm)	15.36 ± .21	15.21 ± .07	.71
Plastron length (mm)	46.63 ± .46	44.28 ± .30	<.01*
Plastron width (mm)	38.88 ± .40	38.32 ± .36	.45
Mass (g)	34.06 ± .31	31.08 ± .28	<.01*
Hatchling fitness:			
Self-righting time (s)	17.53 ± 3.40	18.33 ± 2.77 ($n = 30$)	.34
Righting propensity	5.91 ± .06	5.50 ± .28	.32
Crawl speed (cm/s)	8.87 ± .38	7.34 ± .41	<.01*
Swim speed (cm/s)	14.65 ± .43	14.41 ± .44	.71

*$P < 0.05$.

hypoxia has not been thoroughly investigated, and there are no clear explanations.

We included the hyperoxic treatment to investigate whether increased oxygen availability would positively influence the number of embryos successfully recommencing development following preovipositional arrest or whether it would have some adverse effect on embryonic success and hatchling fitness, in order to test its possible use as a management tool. We found that the number of eggs to break preovipositional arrest and the time taken to recommence development was similar between hyperoxic and normoxic treatments. A likely explanation for this finding is that early embryo mortality (during preovipositional arrest) in flatback turtles is uncommon compared to other sea turtles such as the leatherback, in which this is frequent (Bell et al. 2004; Santidrián Tomillo et al. 2009; Rafferty et al. 2011), and, therefore, there was limited opportunity to detect a beneficial effect of hypoxia.

Marine sea turtle embryos are laid as arrested gastrulas, which coincides with stage 6 of Miller's (1985) 31-stage developmental chronology. Some sea turtle species or populations, such as the critically endangered leatherback turtle, experience a high rate of early embryonic death at stage 6 because eggs fail to recommence development after oviposition (Bell et al. 2004). While nearly all the flatback eggs in our study progressed past this point to at least stage 8 (where white spot formation has occurred), 79.4% of the 34 unsuccessful eggs in the hypoxic treatment died in the early stages of embryonic development. Thus, it appears that eggs in the hypoxic treatment were particularly vulnerable to developmental failure during early development. Certain stages of embryonic development in sea turtles (Miller's stages 6, 22, and 30) have been identified as critical developmental points, with stage 22 being the period of most rapid growth and organogenesis (Bell et al. 2004). Perhaps once development has reached a certain point, embryos may no longer be vulnerable to the effects of extended exposure to hypoxia. These results suggest that embryos may be particularly sensitive to hypoxia during the early stages of development and that there may be some kind of physiological constraint on the period of time that embryos can tolerate hypoxia.

Subsequent embryonic development was also affected following prolonged preovipositional arrest, evident by decreased hatching success in the hypoxic treatment compared to both the normoxic and hyperoxic treatments. This may indicate that flatback embryos are limited in the period of preovipositional arrest that they can withstand before there are negative effects on survival. Conversely, embryo mortality may have occurred as a result of the artificial treatment environment during the initial 5 d postoviposition. However, similar environments were experienced by eggs in both hypoxic and hyperoxic treatments, but hatching success remained high in hyperoxia.

Results from this study highlight that prolonged periods of preovipositional arrest can influence hatching size. Hatchlings from the hypoxic treatment had a significantly larger mean carapace length, carapace width, and plastron length than hatchlings arising from the normoxic treatment. There was also a significant difference in hatchling size between clutches, which is consistent with previous studies (Steyermark and Spotila 2001*b*; Ischer et al. 2009; Read et al. 2012). The production of larger hatchings at a higher cost (greater embryonic death) could be a trade-off that flatback turtles face when egg retention and preovipositional arrest are prolonged. Perhaps the surviving eggs are conditioned in some way while in the hypoxic environment to better withstand nest variables (e.g., fluctuations in oxygen gas concentration or temperature), thus producing bigger hatchlings. Larger hatchlings may have a greater chance of survival because they may be too large or

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strong for predators (such as some birds and crabs) that are gape limited and unable to predate on larger hatchlings (Gyuris 2000; Janzen et al. 2000).

Self-righting responses may have ecological implications for hatchlings during the critical stages following nest emergence and movement down the beach to the ocean. Hatchlings that cannot right themselves quickly may be exposed to predation for longer periods or may dehydrate or overheat (Steyermark and Spotila 2001*a*). No previous studies have investigated the effect of prolonged preovipositional arrest on hatchling fitness. Our findings suggest that extended exposure to hypoxia has no significant effect on hatchling self-righting time or propensity and thus presents no disadvantage in natural settings. We also observed no clutch effects on self-righting response, which is consistent with other studies on green sea turtle hatchings (Booth et al. 2013) and freshwater smooth softshell turtles (*Apalone mutica*; Ashmore and Janzen 2003). On the other hand, studies on freshwater snapping turtles (*Chelydra serpentina*) have found that self-righting times were significantly different between clutches (Steyermark and Spotila 2001*a*). However, in this latter study the lag time (time taken before the hatchling begins to attempt to right itself) was different between clutches rather than the actual mechanical righting time (Steyermark and Spotila 2001*a*), which is consistent with our findings.

Like self-righting ability, the crawling and swimming capacity of turtle hatchlings can have significant implications for their chance of survival in the wild. Hatchlings have no real defense against larger predators and generally do not show evasive behavior when swimming (Gyuris 1994). Thus, the faster that hatchlings can crawl across the beach and swim out to the open waters, the less time they are exposed to potential predators such as birds, crabs, or near-shore fish (Bustard 1972; Ischer et al. 2009). Clutch effects have previously been shown to affect hatchling locomotory ability (Ashmore and Janzen 2003; Booth et al. 2013), which is consistent with our results. However, in relation to extended preovipositional arrest, we found no difference in crawling or swimming speed between hypoxic and normoxic treatments. This indicates that extended egg retention does not appear to have any negative ecological implications for flatback hatchlings because they swim and crawl no differently from hatchlings from the normoxic treatment. Hatchlings from the hyperoxic treatment crawled significantly faster but swam significantly slower than the hatchlings in the normoxic treatment. This result does not give a clear indication as to whether hyperoxia benefits the hatchlings in terms of fitness, especially as the differences in hatchling speed were small (about 2 cm/s, which over 20 m of beach on Bare Sand Island would equate to approximately a 1-min decrease in time spent crawling). Therefore, while our results do not provide evidence that hyperoxia can increase developmental success in this species, they do show that hyperoxia causes no detectable adverse fitness consequences, and so creation of a hyperoxic environment remains worth investigating as a potential action for improving hatching success in populations with high embryonic mortality, such as the Pacific leatherback turtles (Rafferty et al. 2011; Reina et al. 2002), which are at risk of extinction (Spotila et al. 2000).

In conclusion, this is the first study to investigate the effects of prolonged preovipositional developmental arrest on both embryo development and survival, in addition to hatchling size and fitness. It brings us a step closer to understanding the implications of this developmental phenomenon and the impact it has on embryo survival and population persistence. Future understanding of the mechanisms that control preovipositional arrest and recommencement of embryonic development, particularly during early developmental stages, may help us to better design conservation and management strategies to improve hatching success in sea turtle populations globally.

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