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Hypoxia as a novel method for preventing movement-induced mortality during translocation of turtle eggs

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ABSTRACT

Relocation of turtle eggs for research or conservation purposes is associated with significant risk, because they are prone to movement-induced mortality resulting from damage to embryonic membranes. Hypoxic incubation of eggs after oviposition maintains embryos in pre-ovipositional embryonic arrest and delays development. Whether or not this extended developmental pause also delays the onset of sensitivity to movement-induced mortality remains unknown. In previous studies eggs have been incubated in hypoxia using heavy and expensive Perspex chambers. We tested whether extending pre-ovipositional embryonic arrest through hypoxic incubation protects embryos from movement-induced mortality and we investigated more practical and cost-effective methods for transporting eggs under hypoxic conditions. Olive ridley sea turtle (*Lepidochelys olivacea*) eggs were randomly divided among four different treatments after oviposition; a control (normoxic) treatment, Perspex containers or ziplock bags filled with nitrogen gas, or vacuum-sealed bags. Eggs remained in their respective treatment for three days before being removed from their container or bag and placed into artificial incubators. Some eggs from each treatment were inverted when removed from their respective treatment in order to test their susceptibility to movement-induced mortality. We found a reduction in hatching success in the hypoxic treatments (20–43%) compared with the control (68%). However, all methods of hypoxic incubation delayed development and protected against movement-induced mortality. We conclude that plastic bags filled with nitrogen or vacuum bags can be used for maintenance of hypoxia in turtle eggs, thus providing a simple and cost-effective method for transportation of eggs for conservation and research purposes.

1. Introduction

Pressures resulting from the Anthropocene Epoch, such as rapid climate change, habitat loss and fragmentation, are increasing the need for species translocations (Hoegh-Guldberg et al., 2008; McDonald-Madden et al., 2011; Dirzo et al., 2014; Seddon et al., 2014; Mitchell et al., 2016). Translocations can reintroduce species to their former range, augment existing populations, or even assist colonization of suitable new habitat (Dirzo et al., 2014; Seddon et al., 2014). Past translocations of reptiles (excluding sea turtles) have mainly involved translocating adults and juveniles, which has been largely unsuccessful (Dodd and Seigel, 1991; Germano and Bishop, 2009). It has been suggested that translocation of eggs is a more effective strategy (Germano and Bishop, 2009) and it is anticipated that relocation of eggs will be increasingly used for translocating oviparous species (Mitchell et al., 2016).

Sea turtle egg translocation has been used with success in various conservation programs worldwide (Eckert and Eckert, 1990; Pfaller et al., 2009; Hamann et al., 2010). Conservationists often relocate nests laid in compromised locations to areas on the beach considered to have better prospects for high hatching success (Wyneken et al., 1988; Eckert and Eckert, 1990; Garcia et al., 2003; Kornaraki et al., 2006; Pfaller et al., 2009; Pintus et al., 2009; Tuttle and Rostal, 2010; Sieg et al., 2011). In some situations, eggs are even relocated to entirely different coastlines to improve recruitment or to recover populations. For example, from the 1970s to 1980s, thousands of eggs of Kemp's ridley sea turtle (*Lepidochelys kempii*, the most endangered species of sea turtle) were translocated biannually, from Rancho Nuevo, Mexico to Texas, U.S.A., for incubation and subsequent head-starting of hatchlings (Caillouet et al., 2015; Shaver et al., 2016). More recently, large

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numbers of sea turtle eggs were also moved from the Gulf of Mexico Coast to the Atlantic Coast in Florida in response to the Deepwater Horizon oil spill (Safina, 2011). It has even been suggested that more egg relocations to hatcheries and incubators could be necessary to combat the effects of rapid climate change (Fuentes et al., 2012). Researchers also transport eggs for scientific purposes, sometimes to laboratories thousands of kilometres away (Harry and Limpus, 1989; Rafferty et al., 2013; Rafferty and Reina, 2014: Pike et al., 2015).

Given the frequency, and predicted increase, of egg transportation for research and conservation, it is important that the process is efficient and safe. However, the practice of egg relocation for many oviparous reptiles (including all turtles and crocodilians) comes with the risk of movement-induced mortality of the embryo (Limpus et al., 1979; Miller and Limpus, 1983; Chan et al., 1985; Chan, 1989). Movement-induced mortality can occur if eggs are rotated or jolted between approximately 12 h and 20 days after oviposition, with the most vulnerable time varying according to species (Limpus et al., 1979; Parmenter, 1980; Miller and Limpus, 1983; Ferguson, 1985; Deeming, 1991). The start of this period of sensitivity is linked to the breaking of pre-ovipositional embryonic arrest and the fusion of embryonic membranes to the inner surface of the shell (Blanck and Sawyer, 1981; Ewert, 1985; Thompson, 1985; Booth, 2000). Pre-ovipositional embryonic arrest occurs in all turtle species while the eggs are in the oviduct (Ewert, 1985; Miller, 1985; Ewert, 1991; Ewert and Wilson, 1996; Booth, 2000, 2002; Rafferty and Reina, 2012) and the arrest is broken by the increase in oxygen availability when the egg transitions from the hypoxic oviduct to the normoxic nest environment (Kennett et al., 1993; Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). When the arrest breaks and the embryo develops past the gastrula stage and begins neurulation, the vitelline membrane attaches to the shell membrane at the top of the egg (Blanck and Sawyer, 1981; Thompson, 1985; Booth, 2000). If the egg is rotated or jolted after this occurs then the membranes can rupture and the embryo subsequently dies (Blanck and Sawyer, 1981; Deeming, 1991). After approximately 20 days of development the embryo and its membranes have grown large enough for the egg to be moved without mortality (Deeming, 1991).

Currently, the standard protocol to minimize movement-induced mortality during transportation of substantial duration involves lowering the egg temperature to between 4 and 10 °C to slow the rate of embryonic development (Miller and Limpus, 1983; Harry and Limpus, 1989). However, it can be logistically difficult to maintain eggs within this temperature range, especially when working in remote locations with limited facilities. Furthermore, chilling can result in mortality if the temperature of the egg is not maintained appropriately. Therefore, we recently suggested that maintaining eggs in hypoxia may be a viable method for protecting against movement-induced mortality (Williamson et al., 2017). Because pre-ovipositional embryonic arrest does not break until there is an increase in oxygen availability to the egg, it is possible to extend the arrest by placing the eggs into a hypoxic environment after oviposition (Kennett et al., 1993; Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). Extending the arrest delays embryonic development and the fusing of membranes to the eggshell (Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). We hypothesise this would also delay the sensitivity to movement-induced mortality.

Previous studies have shown that placing eggs under water or in nitrogen is an effective way to maintain the eggs in a hypoxic environment and extend pre-ovipositional embryonic arrest (Kennett et al., 1993; Fordham et al., 2006, 2007; Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). Extending arrest after oviposition has been reported to have either; no impact on hatching success (*Chelodina oblonga* and *C. longicollis*, Kennett et al., 1993; *Chelonia mydas*, Williamson et al., 2017), a negative impact on hatching success (*Chelodina colliei*, *C. longicollis*, *Emydura macquarii*, and *Chelonia mydas*, Rafferty et al., 2013; *Natator depressus*, Rings et al., 2015), or a positive impact on hatching success in a freshwater turtle species adapted to laying its eggs under water (*C. oblonga*, Fordham et al., 2006, 2007). The typical method used for extending arrest has involved placing eggs into Perspex (clear polymethyl methacrylate plastic, also called Plexiglass or Lucite) chambers which are then filled with nitrogen to exclude any oxygen (Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). However, these chambers are expensive and cumbersome for use in the field, restricting their suitability for routine use by researchers and conservationists. This method also requires a cylinder of nitrogen or some other inert gas at the nesting site, which is both expensive and impractical in remote field locations.

The aims of our study were to (i) assess whether extending pre-ovipositional arrest after oviposition protects turtle embryos from movement-induced mortality, and (ii) to identify a simple and affordable method for maintaining eggs in arrest after oviposition. We compared two novel methods against the established method of using Perspex chambers (Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). One of the novel methods employed ziplock bags filled with nitrogen, removing the need for the expensive and heavy Perspex chambers. The other method involved the use of vacuum-sealed bags, so neither the Perspex chambers nor the nitrogen cylinders were required. We compared these methods for extending arrest and preventing movement-induced mortality in eggs of the olive ridley sea turtle (*Lepidochelys olivacea*).

2. Materials and methods

2.1. Regulatory approval

All experimental procedures were approved by Monash University's School of Biological Sciences Animal Ethics Committee (Approval BSCI/2015/10). Field research was conducted under a scientific permit issued by the Costa Rican Ministerio Del Ambiente y Energia (MINAE), Sistema Nacional de Áreas de Conservación, Área de Conservación Tempisque (RESOLUCIÓN No ACT-OR-DR-085-15).

2.2. Egg collection

A total of 303 eggs were collected from nesting olive ridleys during two separate arribada nesting events at Playa Ostional, Costa Rica. Eggs were collected into plastic bags using gloved hands. The first group of eggs ($N = 228$) were collected from two nesting females ($n = 120$ and 108) between 17:15 and 17:25 on the afternoon of the 9th of October 2015. The second group of eggs ($N = 75$) were collected on the 7th of November 2015 from four nesting females ($n = 20$, 20, 15 and 20) between 16:00 and 16:15. Eggs were quickly (< 5 min) transported a short distance (< 1 km) from the nesting site to the MINAE station at Ostional. The eggs were individually numbered using a soft pencil to allow traceability of clutch and divided among four treatment groups as described below. The time between oviposition and placement of the eggs into their respective treatments varied from 30 to 50 min.

2.3. Experimental treatments

Eggs were randomised to either a normoxic control treatment ($N = 78$) or one of three hypoxic experimental treatments; a “Perspex” treatment ($N = 75$), a “Ziplock” treatment ($N = 71$), and a “Vacuum” treatment ($N = 79$). The eggs in the control treatment were placed into sand in incubators (described below) and kept in normoxia (~21% O_2) for the duration of incubation. The eggs in the Perspex treatment were placed onto mesh wire sitting above 10 mL of distilled water within Perspex chambers (Resi-Plex Plastics, Vic, Australia), as described previously (Rafferty et al., 2013; Rings et al., 2015; and Williamson et al., 2017). The eggs in the ziplock treatment were placed on mesh wire sitting above 10 mL of distilled water in plastic containers with no lid. The plastic containers were then enclosed within ziplock bags (Ziploc, United States). The ziplock bags and Perspex chambers had in-

Fig. 1. Eggs placed in each of the three hypoxic treatments. From left to right; a Perspex chamber, a vacuum-sealed bag, and a ziplock bag. The Perspex chambers and ziplock bags were filled with nitrogen to exclude oxygen.

flow and out-flow valves at opposite ends of the chamber/bag. The ziplock bags and Perspex chambers were then sealed and 100% industrial grade nitrogen gas (INFRA G.I., San Jose, Costa Rica), humidified by pumping it through a water chamber, was pumped through each bag and chamber for 3 min at a flow rate of 8 L min^{-1} (Fig. 1). To ensure each vessel had reached approximately 1% oxygen v/v (PO_2 ~8 mm Hg) an oxygen sensor and data collection device (Pasco, Roseville, CA) was used to monitor the partial pressure of oxygen of the gas exiting the out-flow valve. Eggs in the vacuum treatment were placed into a vacuum sealable bag (AirLock, Australia) which was then sealed and a hand pump vacuum (Airlock, Australia) was used to create a vacuum within the bag (Fig. 1). Once eggs were in their respective treatments they were transported by car for approximately two and a half hours to a laboratory at the headquarters of Parque Nacional Marino Las Baulas.

The number of eggs per vessel varied between 13 and 24, with a total of four vessels per hypoxic treatment (i.e. four Perspex chambers, four ziplock bags, and four vacuum bags were used). Within-treatment differences in vessels were considered to be negligible so this factor was incorporated into the treatment effect for subsequent analyses. Approximately every 24 h, the ziplock bags and Perspex chambers were re-gassed with nitrogen and the vacuum-sealed bags were re-vacuumed. A duration of three days was chosen because it is equivalent to the maximum duration eggs can be held at a lowered temperature (4–10 °C) before they experience a reduction in hatching success (Miller and Limpus, 1983). Half of the eggs from the second collection were also rotated 180° on a horizontal axis at the end of the three-day experimental period. This was done to assess whether each treatment would protect the eggs from movement-induced mortality.

2.4. Egg incubation and hatching

After eggs were removed from their respective experimental treatments they were placed in sand (7% moisture content by mass) within normoxic incubators (GQF HovaBator model 1632; Grandview Management, Baldivis, Australia) set to 28 °C, which is within their thermal tolerance range (Valverde et al., 2010). The eggs were monitored twice daily for the formation of the characteristic opaque white spot, which is the first externally-visible sign of active development occurring within the egg (Rafferty et al., 2013). Any eggs that showed visible signs of embryonic death (abnormal colouration) or fungal growth were removed from the incubators. Due to logistical limitations in the field, on the 28th of October 2015, (i.e. 19 days since oviposition) all the eggs from the first collection were relocated into nests dug in a hatchery nearby (< 400 m) the laboratory. Eggs were relocated 19 days after oviposition because, if they were relocated earlier than approximately 14 days after oviposition, they may have been susceptible to movement-induced mortality (Limpus et al., 1979). The eggs were buried into four nests, one for each treatment group. Eggs from the second collection were maintained in the incubators through to hatching in order to observe hatching. The time and date of pipping (i.e. breaking of the eggshell by the neonate) and hatching (emergence of the neonate from the egg) for each egg was recorded. After each egg had hatched the hatchling was allowed two days to absorb and internalise its yolk before its morphology and fitness were assessed.

2.5. Excavation of nests and embryonic death

The four nests in the hatchery were excavated two days after hatching and the number of unhatched eggs was carefully counted to calculate the hatching success (Miller, 1999). The unhatched eggs from the hatchery excavations and those that failed to hatch in the laboratory were opened and the stage of each 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 stages as described in detail by Rafferty et al. (2011).

Hatching success (%) = (Hatched eggs/Total number of eggs) × 100

2.6. Hatchling morphology and fitness

Hatchling mass (g) was recorded and the head width, straight carapace width and length were measured using digital callipers (mm). The hatchlings' ability to self-right was tested based on a methodology similar to that employed by Booth et al. (2013). Prior to testing the hatchling was placed in an incubator set to 28 °C for 30 min to allow acclimation. The hatchling was then removed from the incubator and placed on its carapace in a bucket with flattened sand. The time taken for the hatchling to start moving was recorded as the lag time. The total time taken for the hatchling to flip onto its plastron was recorded. The self-righting time was calculated as the total time minus the lag time. This process was repeated two more times for each hatchling and the three times were averaged for each hatchling. If a hatchling did not self-right onto its plastron after 2 min that individual trial was abandoned and the hatchling was given a score of 2 min. The hatchling was then allowed a five-minute break before it was tested again.

2.7. Statistical analysis

Hatching success for each treatment group was calculated as the percentage of eggs to hatch out of the total number of eggs in that treatment. Variation among treatment groups in the hatching success and proportion of eggs to form white spots was assessed using Cochran-Mantel-Haenszel (CMH) tests adjusting for clutch identity. Post-hoc analysis of the CMH tests was conducted using pair-wise Bonferroni corrected chi-squared tests with the independent variable being treatment group. Sample sizes were too small to assess variation between treatment groups in the stage of death using a CMH test so a Fisher's exact test was used instead. Fisher's exact tests were used to compare differences in hatching success, proportion of eggs to form white spots and the stage of death, between eggs from the second collection that were rotated and those that were not. Fisher's exact tests were also used to assess differences in hatching success within treatment groups between rotated and non-rotated eggs.

Fligner-Killeen and Shapiro-Wilks tests were used to assess

homoscedasticity and normality of continuous dependent variables of interest. Between-group differences in incubation duration (days), hatchling mass (g), head width (mm), and carapace width and length (mm) were assessed using analysis of variance (ANOVA) with treatment group as the independent factor and clutch identity as a random blocking factor, with post-hoc comparisons determined using Tukey's HSD test. ANOVAs with treatment group as the independent factor and clutch identity as a random blocking factor were also used to assess differences between incubation length (days), hatchling mass (g), head width (mm), and carapace width and length (mm) for turtles hatching from rotated compared to non-rotated eggs. Total time and aerobic time (total time excluding time in hypoxia) to formation of the white spot, and self-righting time of hatchlings all violated normality and were heteroscedastic ($p < 0.05$). Kruskal-Wallis and Nemenyi post-hoc tests were used to assess between-treatment differences in the self-righting time, and the aerobic and total time to formation of the white spot. Differences between rotated and non-rotated eggs for the self-righting time and the aerobic and total time to formation of the white spot were also assessed using a Kruskal-Wallis and Nemenyi post-hoc test. All values are presented as mean ± standard error or, when normality was violated, median (range). Two-tailed values of $p \leq 0.05$ were considered statistically significant. All analyses were conducted using R software (R Core Team, 2013).

3. Results

3.1. Egg development and white spot formation

There was no significant difference between treatments in the proportion of eggs to form white spots ($X^2_{CMH} = 0.69$, df = 3, $p = 0.88$). Only one egg from each of the control, ziplock and vacuum treatments failed to form a white spot, while two eggs failed to form a white spot in the Perspex treatment. However, these eggs still showed evidence of embryonic development when opened. There was also no significant difference between the rotated and non-rotated eggs from the second collection in the proportion of eggs to form white spots (*Fisher's exact test*; $p = 1$). However, there was significant between-group variation in the latency (elapsed time) to white spot formation (H = 232.76, df = 3, $p < 0.0001$). Eggs in the Perspex and ziplock treatments took approximately three days and 6 h longer than the control group to form white spots, which is approximately equal to the three days they each spent in their respective hypoxic environment (Fig. 2a). Eggs in the vacuum treatment took two days and 9 h longer than the control to form white spots (Fig. 2a). After accounting for time spent in hypoxia (aerobic incubation time; total time excluding time spent in hypoxia) there was significant between-group variation in the aerobic latency (total time excluding time in hypoxia) till white spot formation (H = 151.66, df = 3, $p < 0.0001$ Fig. 1b). The Perspex and ziplock groups took approximately 7 h longer in normoxia than the control group to form white spots. However, the aerobic latency till white spot formation in the vacuum group was approximately 13 h less than for the control group. There was no significant difference between rotated and non-rotated eggs from the second collection in latency till white spot formation for both total incubation time (H = 0.42, df = 1, $p = 0.52$) and aerobic incubation time (H = 0.47, df = 1, $p = 0.49$).

3.2. Hatching

There was significant variation in hatching success among the treatment groups ($X^2_{CMH} = 42.65$, df = 3, $p < 0.0001$). The control group had a greater hatching success than all three hypoxic treatments. Hatching success was greater with the use of vacuum bags compared to the ziplock bags filled with nitrogen, while hatching success for eggs incubated in hypoxia in Perspex chambers was not significantly different to that for either the vacuum or ziplock treatments (Fig. 3).

Within the eggs from the second collection the hatching success of rotated eggs was significantly lower than that of non-rotated eggs in the control group only ($p < 0.01$, Fisher's exact test; Fig. 4). There was no significant difference between hatching success for the rotated and non-rotated eggs for the Perspex, ziplock, and vacuum treatments ($p = 0.14$,

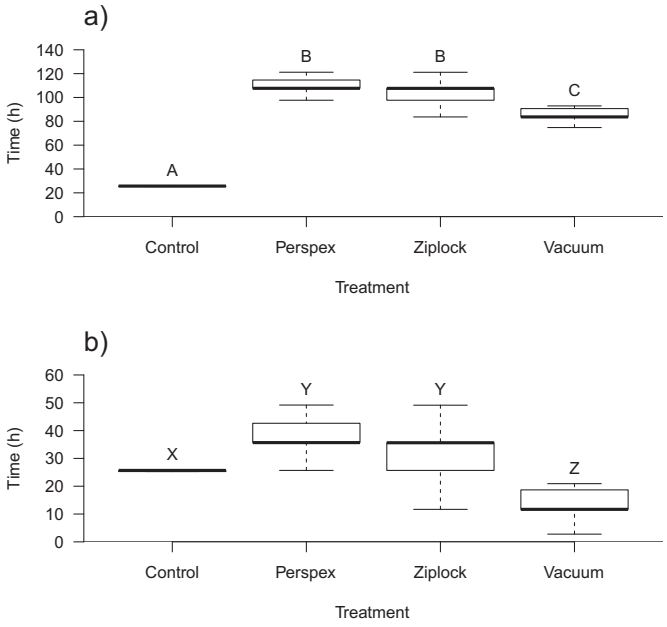

Fig. 2. Latency till formation of the white spot on eggs measured as a) total time, or b) aerobic time. Eggs were subjected to either a control (normoxic) treatment ($n = 76$), or placed into Perspex chambers ($n = 74$) or ziplock bags ($n = 70$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags ($n = 78$). Aerobic time was calculated as total time excluding time spent in hypoxia. Boxplot centre lines show medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. When the letters above each whisker are the same, latency to white spot formation did not differ significantly between corresponding treatment-groups (Kruskal-Wallis and Nemenyi's post-hoc test; $p \leq 0.05$).

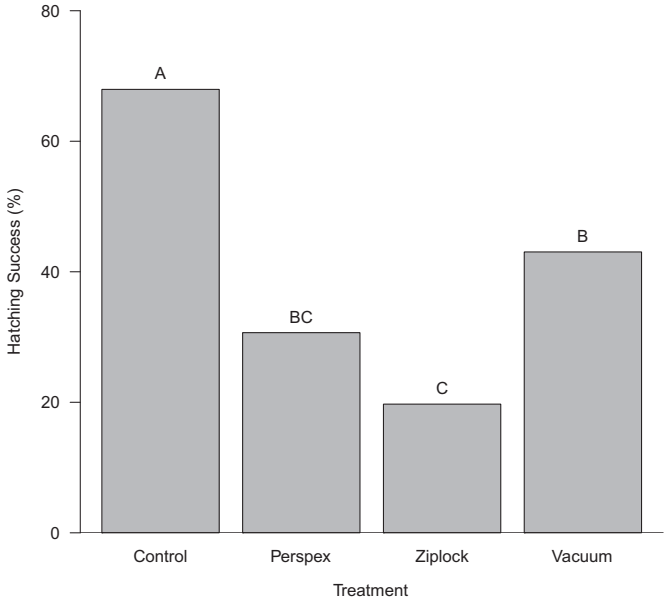

Fig. 3. Total hatching success for olive ridley eggs placed into four treatments. Eggs were placed into either a control treatment ($N = 78$), Perspex chambers ($N = 75$) or ziplock bags ($N = 71$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags ($N = 79$). When letters above each bar are the same, the hatching success did not differ significantly between the corresponding treatments (Bonferroni corrected chi-squared test with six pair-wise comparisons; $p \leq 0.05$).

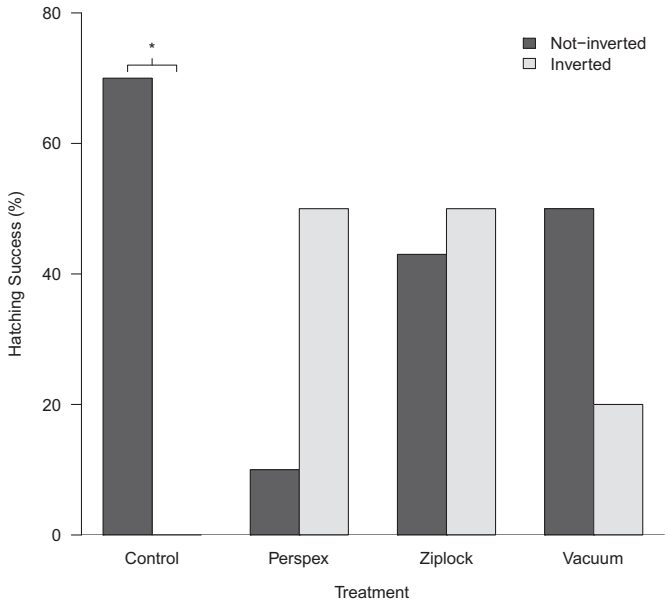

Fig. 4. Hatching success for olive ridley eggs subjected to various treatments and incubated in the laboratory till hatching. Treatments were either control (normoxia; $N = 20$), Perspex chambers ($N = 20$) or ziplock bags ($N = 15$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags ($N = 20$). Eggs remained in their respective treatments for three days before being returned to normoxia. After the three day treatment period, half of the eggs from each treatment (7 of 15 in the ziplock treatment) were rotated to assess differences in movement induced mortality between the treatments. Bar and asterisk (*) above treatment group indicates a significant difference in hatching success between eggs that were or were not inverted 3 days after oviposition according to a Fisher's exact test ($p \leq 0.05$).

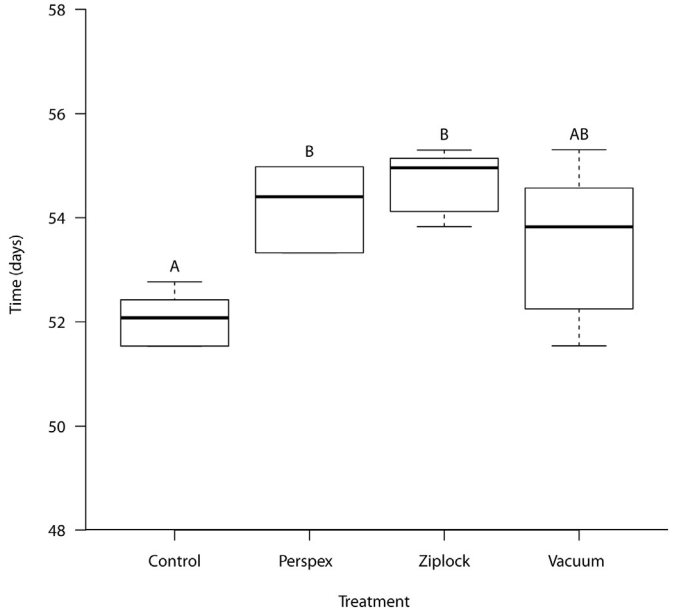

Fig. 5. Incubation length of olive ridley eggs placed into four treatments immediately after oviposition. The four treatments lasted three days and consisted of; 1) a control treatment ($n = 7$), 2) Perspex chambers ($n = 6$) or 3) ziplock bags ($n = 7$) into which nitrogen gas was pumped to exclude oxygen, or 4) vacuum-sealed plastic bags ($n = 7$). These eggs were then maintained until hatching in incubators in the laboratory. Boxplot centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. When the letters above each whisker are the same, latency to white spot formation did not differ significantly between corresponding treatments (Kruskal-Wallis and Nemenyi's post-hoc test; $p \leq 0.05$).

$p = 1$, $p = 0.35$; respective Fisher's exact tests; Fig. 4).

There were between-treatment differences in the total time taken to hatch for the eggs incubated in the laboratory through to hatching ($F_{(3,6)} = 5.18$, $p < 0.05$; Fig. 5). Relative to control eggs, the delay in hatching for the Perspex and ziplock treatments was approximately equal to the three day period those eggs spent in hypoxia. The vacuum treatment had a slightly shorter incubation period than the Perspex and ziplock treatments, taking one and a half days longer to hatch than the control group.

3.3. Embryonic mortality

There were no significant between-treatment differences in the stage that embryos died at (*Fisher's exact test*; $p = 0.36$). Within the eggs incubated in the laboratory until hatching there were also no significant differences in the stage of death of rotated and non-rotated eggs (*Fisher's exact test*; $p = 0.71$).

3.4. Hatchling morphology and fitness

There were no significant between-treatment differences in hatchling mass, head width, carapace width and length, or self-righting time (Table 1). For the eggs that survived to hatching, there was also no apparent influence of egg rotation on hatchling mass (g; $F_{(1,3)} = 0.03$, $p = 0.87$), head width (mm; $F_{(1,3)} = 0.12$, $p = 0.76$), carapace width and length (mm; $F_{(1,3)} = 0.04$, $p = 0.85$, and $F_{(1,3)} = 7.85$, $p = 0.07$, respectively), and self-righting time (sec; $H = 1.66$, $df = 1$, $p = 0.20$).

4. Discussion

Our findings suggest that extending pre-ovipositional embryonic arrest by placing turtle eggs into hypoxia protects embryos from movement-induced mortality. Thus, hypoxia should be a valid method to use for egg transportation and may be preferable to lowering the temperature of the eggs during transportation (Miller and Limpus, 1983; Harry and Limpus, 1989). Our results also confirm that oxygen availability to the embryo controls pre-ovipositional embryonic arrest in turtles (Kennett et al., 1993; Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017). All three hypoxic incubation methods were capable of extending arrest, but this came with a reduced overall hatching success compared to the control. Importantly, our findings indicate that it is not necessary to use nitrogen, because our vacuum bag treatment had the same or greater hatching success than the two nitrogen treatments. Thus, it should be possible for inexpensive vacuum-sealed bags to be used in remote locations and on a large scale by conservation and research groups for future egg translocations.

As we have found previously in other species of freshwater and marine turtle (Rafferty et al., 2013; Rings et al., 2015; Williamson et al., 2017), the developmental schedule, both in terms of formation of the white spot and hatching, was delayed by hypoxic incubation. However, one species of freshwater turtle (*C. oblonga*) has been found to speed up the developmental schedule in response to extended periods of pre-ovipositional embryonic arrest (Fordham et al., 2006, 2007). Our vacuum treatment did not delay development by as much as the nitrogen treatments. An explanation for the difference between the vacuum bags and the other hypoxic treatments in developmental timing could be that air pockets may have remained within the bags or there was some slight leakage after the bags were vacuum-sealed. A specially designed vacuum bag suited for spherical objects would potentially reduce the amount of air pockets and leakage.

The three-day period of hypoxia employed in the current study is probably longer than would be required for transportation of eggs for conservation purposes, even when transporting eggs around the world. We previously found no reduction in hatching success when green turtle eggs were placed in hypoxia for three days (Williamson et al., 2017). However, olive ridleys have one of the shortest incubation periods of all

Table 1
Morphology and fitness of olive ridley hatchlings from the various incubation treatments.

Trait	Control	Perspex	Ziplock	Vacuum	Test statistic	*p*-Value
Mass (g)	14.2 ± 0.6	12.8 ± 0.6	15.6 ± 0.7	15.1 ± 0.9	$F_{(3,6)} = 2.13$	0.20
Head width (mm)	14.7 ± 0.2	13.84 ± 0.27	14.48 ± 0.21	14.2 ± 0.3	$F_{(3,6)} = 2.46$	0.16
Carapace width (mm)	33.7 ± 0.7	30.8 ± 1.1	34.9 ± 0.8	33.8 ± 1.0	$F_{(3,6)} = 2.09$	0.20
Carapace length (mm)	40.7 ± 0.6	37.8 ± 1.1	40.3 ± 0.8	39.8 ± 1.0	$F_{(3,6)} = 2.52$	0.16
Self-righting time (sec)	33 (2 – 120)	17 (3 – 120)	4 (2–29)	6 (1 – 120)	$H_{(3)} = 3.57$	0.31

Eggs were place into either a control treatment ($n = 7$), Perspex chambers ($n = 6$) or ziplock bags ($n = 7$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags ($n = 7$). Data are mean ± standard error, except for self-righting time which violated normality, so is presented as median (range). Differences between groups were assessed using ANOVA. The Kruskal-Wallis test was used for self-righting time, as the data violated normality.

sea turtles (Crastz, 1982; Miller, 1985). Thus, three days represents a larger proportion of development for that species compared to green turtles (Miller, 1985). Extended pre-ovipositional arrest has been shown to compromise early development in a population of leatherback turtles (Rafferty et al., 2011). However, freshwater turtle (*C. oblonga*) eggs held under water for six weeks after oviposition had a greater hatching success than those that were incubated in normoxia immediately after oviposition (Fordham et al., 2006, 2007). A reduction from three to two days spent in hypoxia may improve hatching success for olive ridley eggs. Future studies should explore the relationships between duration of hypoxic incubation and hatching success, to allow species-specific optimization of protocols for the use of hypoxia for translocation of turtle eggs.

While extending embryonic arrest by three days decreased hatching success, we did not detect any impact on hatchling morphology or fitness. Our observations contrast with those of Rings et al. (2015), who found that flatback turtles incubated in hypoxia to extend arrest for five days were larger and swam faster than a control group. Potentially, the difference in the amount of time the arrest was extended for (an extra two days) may explain why our results differ. However, extending arrest after oviposition in a freshwater turtle (*C. oblonga*) has been found to be negatively correlated with post-hatching survival (Fordham et al., 2007). Taken collectively, these results suggest that extending embryonic arrest can have mixed consequences for hatchling fitness and requires further investigation for specific species.

The maximum time eggs can be held at a lowered temperature for transportation, while avoiding large reductions in hatching success, is three days (Miller and Limpus, 1983). This period is comparable to what we have shown with hypoxia. But hypoxia is likely more suitable for situations in which the equipment required for chilling is not available. Nevertheless, eggs should still be kept from reaching extreme temperatures while in hypoxia. If egg temperature drops to close to freezing point, or if the eggs become too hot (> 35 °C) during transportation, it is likely that the embryos will not successfully develop once they are removed from hypoxia. Ideally temperature should be closely controlled during transportation, regardless of the method used to extend embryonic arrest. Future studies should be designed to investigate the success of eggs that are both chilled and kept in hypoxia during transportation. Other methods for maintaining eggs in hypoxia could also be investigated, such as submersion under water or covering individual eggs with a biofilm or plastic wrap.

The utility of using vacuum-sealed bags extends beyond avoiding the need for nitrogen gas cylinders for maintaining hypoxia. The bags are relatively cheap (US$1–5 per bag) in comparison to the Perspex chambers (~$100 per chamber) and the equipment required for chilled transportation (minimum $100 per cooler box with sufficient ice packs). Vacuum bags can easily be reused after sterilisation. The eggs are tightly sealed in place as well, which further reduces the probability of eggs rotating during translocation. The need to translocate eggs for conservation purposes is predicted to increase (Fuentes et al., 2012) and sustained hatchling production is a global priority (Rees et al., 2016). Thus, use of hypoxia to extend development arrest, particularly with the relatively simple and inexpensive approach of using vacuum-sealed bags, provides a valuable tool for conservationists and researchers. The utility should be universal to all species where pre-ovipositional embryonic arrest occurs; all turtles, chameleons and tuatara (Rafferty and Reina, 2012). Indeed our vacuum-sealed bag transportation technique has already been used on a North American freshwater turtle species to successfully protect against movement-induced mortality during transportation by car for a period of 38 h (J. Wyneken, personal communication).

In conclusion, our observations suggest that extending embryonic arrest by placing eggs in hypoxia protects embryos from movement-induced mortality. We also present evidence that vacuum-sealed bags can be used to extend arrest, in a cost-effective and convenient manner, and so allow safe transportation of turtle eggs without the need for gas cylinders or chilling equipment. This improves our capability to maximise positive outcomes during research and conservation practices involving egg translocation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2017.10.009.

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