CONTROL AND ECOLOGICAL SIGNIFICANCE OF EMBRYONIC DEVELOPMENT IN TURTLES AND CROCODILES

Sean A. Williamson

Control and ecological significance of embryonic development in turtles and crocodiles

Sean Alexander Williamson

BScAdv Hons (University of Wollongong)

A thesis submitted for the degree of *Doctor of Philosophy* at

Monash University in 2017

School of Biological Sciences

Table of Contents

List of Figures

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Chapter Six

Chapter Seven

List of Tables

Abstract

Crocodilian and turtle eggs are laid at an early stage of embryonic development. In turtles, embryonic development arrests at the gastrula stage within the mother's oviducts. Arrest is maintained by low oxygen availability in the oviduct and is broken upon exposure to normoxia at oviposition. It provides flexibility in the reproductive schedule, with some turtle species capable of delaying nesting by weeks or even a whole year. However, embryonic arrest can also impair reproductive success, being implicated as a potential cause for poor hatching success of leatherback turtles (*Dermochelys coriacea*). I investigated the physiological processes and ecological and evolutionary significance of embryonic arrest in turtles, and whether this phenomenon also occurs in crocodilians.

To determine the latency between oviposition and the breaking of embryonic arrest, green sea turtle (*Chelonia mydas*) eggs were incubated under hypoxic condition for three days, commencing between 30 minutes to 48 hours after oviposition. Provided eggs were placed into hypoxia within 12 hours of oviposition, hatching success did not differ from the control treatment, but after longer periods the hypoxic incubation resulted in embryonic death. I conclude that embryonic arrest is not broken until at least 12 hours after oviposition, coinciding with the time at which turtle eggs become susceptible to movement-induced mortality.

Movement-induced embryonic mortality occurs when eggs are jolted or rotated between approximately 12 hours and 20 days after oviposition. I tested whether extending embryonic arrest using hypoxia would protect against movement-induced mortality and whether this could be achieved using practical and cost-effective methods. After oviposition, olive ridley sea turtle (*Lepidochelys olivacea*) eggs were placed into one of four treatments for three days; a control

(normoxic) treatment, vacuum-sealed bags, or containers filled with nitrogen gas. Some eggs from each treatment were inverted upon removal from their treatment to assess susceptibility to movement-induced mortality. All three hypoxic treatments protected eggs from movement-induced mortality, whilst none of the control eggs that were inverted survived. I conclude that vacuum-sealed bags or plastic bags filled with nitrogen provide a cost-effective and efficient method for safe transportation of eggs for conservation and research purposes for up to three days post-oviposition.

I tested whether crocodilian eggs exhibit pre-ovipositional embryonic arrest by incubating saltwater crocodile (*Crocodylus porosus*) eggs in hypoxia for three or six days immediately after oviposition. I also examined whether increased oxygen availability (hyperoxia; 42% oxygen) had any detectable impact upon hatching success or hatchling fitness. Hypoxic incubation did not delay embryonic development, but markedly reduced hatching success. Hatching success and hatchling fitness in the hyperoxic treatment did not differ from the control. The absence of pre-ovipositional embryonic arrest in crocodilians implies that they are unable to delay nesting to avoid adverse environmental conditions. The lack of a negative effect of hyperoxia on embryonic development provides impetus for studies to determine whether hyperoxic incubation could improve the low hatching success of some endangered species such as leatherback turtles.

To determine whether hyperoxia could improve hatching success of leatherback turtles, eggs were incubated in 42% oxygen for five days after oviposition. No effects of hyperoxia on early stage death or hatching success were observed. However, hypoxia extended arrest in this species. Furthermore, the longer the period of extended arrest (three vs five days in hypoxia), the lower the hatching success. Failure to resume development after embryonic arrest has a negative

impact on the reproductive success of leatherback turtles, but hypoxia does not appear to be the cause.

To examine differences in embryonic arrest of olive ridley eggs laid during a mass-nesting (arribada) compared with a solitary-nesting event, eggs were maintained in hypoxia for between 3 and 30 days after oviposition. Developmental arrest was maintained by hypoxia in eggs laid during an arribada for up to 15 days, with little impact on hatching success. In contrast, eggs laid during a solitary nesting event could not be maintained in arrest by hypoxia for more than 4 days without a detrimental effect on hatching success. Thus, the reproductive strategy of the mother is associated with differing capacity for extending pre-ovipositional developmental arrest in arribada vs solitary nesting females.

In summary, these studies show that (i) breaking of embryonic arrest is linked to the start of the embryo's susceptibility to movement-induced mortality, (ii) turtle eggs can be protected from movement-induced mortality using plastic bags filled with nitrogen or vacuum bags; simple methods that should be applicable to any species that exhibits pre-ovipositional embryonic arrest, (iii) crocodilians do not arrest development prior to oviposition, (iv) extended arrest negatively impacts hatching success in leatherback turtles and hyperoxic incubation does not improve hatching success, and (v) reproductive strategy influences tolerance to extended embryonic arrest in eggs of olive ridley turtles.

Copyright Notice

© Sean Williamson (*2017*).

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

Thesis Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers published in peer reviewed journals (*Physiological and Biochemical Zoology* and *Biological Conservation*) and one publication currently in press (*Royal Society Open* Science). The core theme of the thesis is *control and ecological significance of embryonic development in turtles and crocodiles*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Roger Evans and Richard Reina. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. This thesis also includes four chapters which have not been submitted for publication (Chapters 1, 4, 5, and 7). In the case of *Chapters 2, 3, 6, Appendix C & D* my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Extent of student contribution
2	When is embryonic arrest broken in turtle eggs?	Published in *Physiological and Biochemical Zoology*	Concept, collecting and analysing data and writing first draft
3	Hypoxia as a novel method for preventing movement-induced mortality during translocation of turtle eggs.	Published in *Biological Conservation*	Concept, collecting and analysing data and writing first draft
6	Ecological and evolutionary significance of a lack of capacity for extended developmental arrest in crocodilian eggs.	In press in *Royal Society Open Science*	Concept, collecting and analysing data and writing first draft
App. C	Hematology and serum biochemistry for free-ranging freshwater crocodiles (*Crocodylus johnstoni*) in Western Australia.	Published in *Journal of Wildlife Diseases*	Ethics, permits, data collection and project coordination
App. D	Plastic fork found inside the nostril of an olive ridley sea turtle.	Published in *Marine Turtle Newsletter*	Ethics, permits and data collection

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 17/12/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 17/12/2017

Acknowledgements

First and foremost, I would like to thank the 'supermen', my two supervisors Richard Reina and Roger Evans. You have both been outstanding in supporting me throughout my project and helping me develop as an independent researcher. Through your encouragement and guidance, my ability to think critically and communicate these thoughts has improved greatly.

Richard, thank you for accepting a kid from 'the gong' to come and discuss potential PhD ideas with you. My initial penguin project idea might not have been great but you were willing to suggest I work on sea turtles in Costa Rica, a very hard sell! I came to your lab upon a recommendation that you are one of Australia's leading ecophysiologists. I leave your lab convinced of that. Your insights and ability to mentor students on a range of topics across a wide variety of taxa is very impressive. Thank you sincerely for introducing me to the sea turtle research community, where I found inspiration and friendship. Lastly, thank you for providing an inspiring example for balancing academic and home life. Lene, Max and Felix are extremely fortunate and I wish the boys all the success for their future. You also supported my own 'home life' and allowed me to follow Chenae to Canberra and introduced me to Paul Cooper at ANU.

Roger, thank you for agreeing to co-supervise me on what has turned out to be a very different research project to what you might have previously supervised and to what we originally intended on carrying out. Even though there might not be any Oxylite data in my thesis, I am very grateful you entrusted me with taking the machine into dusty and remote croc country. I will be forever grateful of Tony for putting together the 'turtle oxygen research team'. You have gone above and beyond with your supervisory work, always providing me with prompt and valuable comments on my writing. Your honest and detailed feedback has truly transformed my

approach to writing, this is something I will always appreciate and has me feeling well armed for my future writing endeavours. Finally, thank you for providing me with inspiration for a commitment to the scientific method and to uncovering the truth. Hopefully one day in the future I can join Mandy and yourself for a walk through the hills of India!

Chenae, you have stood by me and supported me every step of the way throughout this crazy PhD journey. From helping out at the croc farm in Darwin, to sleepless nights placing turtle eggs into hypoxia on Heron Island, patrolling the beach for turtles and helping with experiments in Costa Rica, you have always been there to help. I am thankful you were so patient during this long journey, especially during the periods where we were apart for months on end. You have provided me with strength and stability over the course of this journey. You always managed to talk me out of despair when things were not working out. There is so much of this thesis that was dependent upon your help and ideas. You have been my draft checker, field assistant, egg checker, turtle tagger and water dragon whisperer. Forever and from the bottom of my heart, thank you!

To my friends, thank you and damn you for always providing many distracting opportunities for fun adventures over the years. Adam and Connor, Ben and Bianca, Brent, Browny, Isaac, Jack and Amy, Jordan, Korber, Rachel, Reesy, Tarant, Todd and Emily, you are all truly legends and I'll be more present from here on hopefully. Particular thank you to Adam, Ben, Isaac, Reesy and Tarant for helping out in the field, whether chasing crocs, dragons or turtles. I hope you had as much fun as me. I have had the pleasure of sharing a house with quite a few housemates over the course of my PhD. Angus, Anna, Bec, Elyshia, Julia, Louisa, Mischa, Myles, Nicki, Rhys, Samichya, Simon and Torqhuil, you have all been great to live with and so supportive, thank you!

My family have always supported my endeavours throughout life and this was no exception over the last four years. Mum, your humility and commitment to your research and professional obligations is an inspiration. Dad, thank you for being so supportive throughout this period and always being there to listen. I also need to state a written thanks for carrying suitcases of equipment halfway around the world for me! Both of your support and love has been amazing and has honestly been so important in getting me through. I am forever thankful. My extended family around the world, both Forsyths and Williamsons, have always been keen for a laugh and offered this poor grad student a couch on many an occasion, you are all brilliant people, thank you all for what you have shared!

Thanks to my Monash family for many fun nights and interesting conversations. Amanda, Clem, Evatt, Karen, Krys, Lotte, Manoj, Marcus, Michael, Pat, Rowan, Stephen and Will, thanks for providing the laughs. I was fortunate enough to be elected to the Postgraduate Committee by the student body. It was great working with the other student representatives to attempt to provide an enriching academic and social experience for everyone within the School of Biological Sciences. My Monash and 'Reina lab' experience would have been appreciably worse without the support and friendship of the other Reina lab students, Brian, Cathy, Chloe, Chris, Derek, Fran, Leo, Licia, Lynette, Nikki, Sonia, and Tony, thank you all! Fran we may have failed at our croc endoscopies but that was some pretty amazing fieldwork at Windjana Gorge nonetheless. Special thanks to Tony Rafferty, you shared your knowledge, insights and connections with me and really helped get my project underway.

I have been fortunate to work with a wide range of institutions and collaborators throughout Australia during this project. Firstly, Grahame Webb, Charlie Manolis and the staff at Crocodylus Park welcomed me to work with them in Darwin at the start of my PhD. This

experience will live with me forever and always be a source for great stories to tell. A particular shout out to the angry male ‘salties’ who were so welcoming when I entered their pens to collect eggs. Thank you to Ruchira Somaweera and Tracy Sonneman for inviting Fran Scheelings and myself to join you and the Department of Parks and Wildlife staff and volunteers on the freshwater crocodile survey of Windjana Gorge. I would also like to express my gratitude to Peter Harlow at Taronga Zoo for sharing his extensive knowledge of water dragon nesting and reproduction. My fieldwork and experiment conducted at Heron Island would not have been possible without help from Danielle Annese and Elizabeth Perkins and the staff of Heron Island Research Station, thank you all. Thanks also to Janine Ferguson and the Queensland National Parks Turtle Volunteers, in particular Jasmine Burgan, Zoe Truscott, and Tessa Horgan, for assistance with egg collections and letting me assist with some of their work. John Guy from Good Food Solutions kindly catered for my second stay on the island.

I was also fortunate to collaborate with a range of international researchers and NGOs for my Costa Rican fieldwork. Frank Paladino, Bibi Santidrián Tomillo, George Shillinger and Jim Spotilla welcomed me to work with them and the Leatherback Trust in Playa Grande. I am very appreciative of the support offered by Jenell Black, Brett Butler, Lauren Cruz, Lia Domico, Tera Dornfeld, Lauren Hackney, Collin Hertz, Lindsay McKenna, Nathan Robinson and Eli Solano during my stay at Playa Grande. Thank you to all the Earthwatch Institute volunteers for assistance as well. Yudi Arguello- Gómez and Carlos Orrego were very helpful with my egg collections from Playa Ostional, thank you both. Thank you to Carlos Fernández and all the staff at Ecology Project International and Pacuare Nature Reserve for welcoming me to the Caribbean side of Costa Rica and helping with my leatherback experiment. In particular I would like to show my gratitude to Germaine Barrantes, Renato Bruno, Ashleigh Bandimere, Pedro Morales

Chavez, Nerine Constant, Hyeily Hernandez Gonzalez, Nathan Goldschmidt, and Lucy McNaught, for their assistance and friendship whilst at Pacuare. Aimee Hoover and George Shillinger also joined me at Pacuare, and I would like to thank them for allowing me to help out with their hatchling tracking study.

I followed Chenae to Canberra towards the end of this project, and I would like to thank Paul Cooper and Scott Keogh for welcoming me as a visitor to the Australian National University (ANU). Paul, I am so grateful that you offered me some office space in your lab and connected me with some work opportunities. Importantly, you also introduced me to the ANU workshop who helped put together some gas equipment for the fieldwork in Pacuare and that really helped streamline my final experiment. Thank you for also being a great mentor and always available for a laugh.

Lastly, I am very appreciative of the funding offered by many organisations in order for this research project to be carried out. Firstly, thank you to Bill Holsworth and the Holsworth Wildlife Research Endowment for generously providing funding for the completion of my Australian fieldwork. This research was supported by an Australian Government Research Training Program (RTP) Scholarship. The Crocodile Specialist Group of the International Union for Conservation of Nature Species Survival Commission provided me with a student grant to undertake my crocodilian experiments. Thank you to the Society for Experimental Biology for the award of two grants to attend the annual society meeting in Brighton in 2016. Thanks also to the International Sea Turtle Society for twice awarded me a student grant to travel to the annual meetings in Turkey in 2015 and Peru in 2016. Finally, thanks to the Leatherback Trust and Ecology Project International for their in-kind financial support during my fieldwork in Costa Rica.

Chapter 1. General Introduction

1.1 Vertebrate reproduction and embryonic development

Vertebrate colonisation of the terrestrial environment was once constrained by the requirements of reproduction (Kohring 1995), because the amphibians from which terrestrial vertebrates evolved laid eggs that were generally externally fertilised and required oviposition in water in order to develop successfully (Duellman and Trueb 1986; Wake 1993). Fertilisation of the egg then shifted to the female oviduct allowing for the evolution of the amniotic egg through deposition of amniotic membranes (including eggshell) in the oviduct (Blackburn 1998). The evolution of the amniotic egg subsequently preadapted vertebrates for the colonisation of the terrestrial environment by negating the need to reproduce in water (Reisz 1997). Reptiles diversified greatly once they colonised the terrestrial environment (Little 1990) and once on land, viviparity (live-birth) evolved from oviparity (egg-laying) over 100 times within reptilian lineages (Shine 1985, 2005; Blackburn 2006). However, some oviparous species, such as turtles and crocodilians, returned to the aquatic environment, but remained dependent upon returning to terrestrial environments to lay eggs.

Turtles and crocodilians share many life history traits. They take many years to reach maturity, experience low adult mortality and are relatively long-lived species (Castanet 1994; Shine and Iverson 1995). All species are oviparous and during nesting seasons females oviposit large clutches of eggs (2-200) into terrestrial mound or hole nests (Hirth 1980; Bjorndal and Carr 1989; Thorbjarnarson 1996). Eggs, hatchlings and juveniles experience high rates of predation until reaching maturity (Janzen *et al.* 2000; Somaweera *et al.* 2013). However, crocodilians and

turtles differ in the level of parental care. Turtles provide no parental care after oviposition, whilst crocodilians provide some in the form of nest- and crèche-guarding (Shine 1988).

Reproduction in crocodilians and turtles involves copulation and insemination of the female with sperm prior to the first nesting of the reproductive season (Ferguson 1985; Miller 1997). The sperm is then stored in the oviduct (Birkhead and Møller 1993). Follicles are ovulated from the ovary as yolk with accompanying ova. Shortly after, the ova are fertilised by the sperm in the oviduct. Secretion of the albumin (egg-white) and amniotic membranes (eggshell membranes) also occurs in the oviduct. Embryonic development commences after fertilisation and prior to oviposition of the egg (Ferguson 1985; Miller 1985). Embryos of crocodilians and turtles are relatively undeveloped at oviposition, having only reached the gastrulation or neurulation stages of development respectively (Ewert 1985; Ferguson 1985; Miller 1985). After oviposition both crocodilians and turtle embryos are susceptible to movement-induced mortality if eggs are rotated or shaken during approximately the first third of development (Limpus *et al.* 1979; Ferguson 1982; Miller and Limpus 1983; Ferguson 1985; Deeming 1991). However, once in the nest eggs are rarely rotated and eggs generally develop continuously until hatching unless some other factor affects their development.

1.2 Embryonic arrest

Embryonic development across a range of oviparous taxa is not always continuous until hatching. There are various environmental variables that control embryonic arrest, such as temperature, oxygen availability, and moisture (Rafferty and Reina 2012). Vertebrate embryos can pause development at various stages of development (Ewert 1991; Renfree and Shaw 2000; Rafferty and Reina 2012) and there are many potential selective benefits in doing so. Generally,

pausing or arresting allows development to be timed beneficially with environmental and ecological conditions (Rafferty and Reina 2012). Furthermore, hatching can then occur when conditions are most beneficial to the neonate (Ewert 1991; Ewert and Wilson 1996).

1.3 Pre-ovipositional embryonic arrest

All turtle species studied to date arrest development prior to oviposition whilst the eggs are in the oviduct (Risley 1944; Lynn and von Brand 1945; Miller 1982; Ewert 1985; Miller 1985; Ewert 1991). This pre-ovipositional embryonic arrest is maintained by the hypoxic (low oxygen) environment in the oviducts (Rafferty *et al.* 2013). Artificial or experimental incubation of eggs in hypoxia after oviposition maintains the arrest until the eggs are placed into normoxia (normal oxygen levels) (Kennett *et al.* 1993; Fordham *et al.* 2006; Fordham *et al.* 2007; Rafferty *et al.* 2013; Rings *et al.* 2015). Temperature is critical for the developmental rate of reptilian eggs, but temperature does not appear to influence the time at which pre-ovipositional embryonic arrest is broken (Rafferty and Reina 2014). Therefore, the level of oxygen available to the embryo is critical for the functioning of pre-ovipositional arrest and early development in turtles. However, how long embryos require in normoxia before breaking arrest is currently unknown.

Whether, like turtles, crocodilians arrest development prior to oviposition is also unknown, although their developmental patterns are similar. However, there is contention as to whether or not they exhibit pre-ovipositional arrest. Embryos of crocodilians are slightly more developed at oviposition than are those of turtles, suggesting they may not arrest (Ferguson 1985; Ewert 1991; Rafferty and Reina 2012). However, extended retention of eggs by crocodilians has been reported (Reese 1931; McIlhenny 1934; Ferguson 1985), suggesting they may arrest embryos *in*

utero. Experimental clarification of the capacity of crocodilian eggs to arrest development in hypoxia after oviposition is needed.

There are important ecological and evolutionary consequences of pre-ovipositional embryonic arrest. In turtles it affords the mother greater flexibility in her nesting date, allowing her to potentially avoid adverse conditions. For example, some freshwater turtles are capable of delaying nesting for many months (Buhlmann *et al.* 1995), whilst some sea turtles are capable of delaying nesting for many weeks (Plotkin *et al.* 1997). This is an important ability for a taxon that provide no parental care after oviposition. Furthermore, pre-ovipositional arrest in a hypoxic oviduct may mean the evolution of viviparity in turtles is constrained, because further development within the oviduct is unlikely if eggs are retained (Rafferty *et al.* 2013). Whether this is the case for crocodilians as well is currently unknown.

When pre-ovipositional arrest is extended after oviposition through incubation of eggs in hypoxia there can be ramifications for developmental timing, hatching success, and hatchling fitness. Development, including formation of the opaque white spot and hatching, is usually delayed for a period equivalent or greater than the time period the embryo spends in extended pre-ovipositional arrest (Rafferty *et al.* 2013; Rings *et al.* 2015). However, embryos of a freshwater turtle (*Chelodina oblonga*) adapted to underwater nesting are capable of accelerating their developmental schedule following extended periods of arrest (Fordham *et al.* 2006; Fordham *et al.* 2007). Extended arrest can reduce (Rafferty *et al.* 2013; Rings *et al.* 2015) or increase (Fordham *et al.* 2006; Fordham *et al.* 2007) hatching success. Flatback turtle (*Natator depressus*) embryos that had pre-ovipositional arrest extended for five days through incubation in hypoxia resulted in hatchlings that swam faster and were larger than a control group (Rings *et al.* 2015). Extended arrest in a freshwater turtle (*C. oblonga*) had negative impacts of post-hatching

survival (Fordham *et al.* 2007), so there are apparently interspecific differences in the impact of extended arrest on reproductive success in turtles. Furthermore, even though turtles can suspend development to potentially delay nesting through the use of pre-ovipositional embryonic arrest it is clear that there are still important consequences if they over-extend the arrest.

An investigation of a long-term data set on the nesting and reproductive success of leatherback turtles from Playa Grande in Costa Rica found that extended arrest (identified through repeated failed nesting attempts) resulted in increased early-stage embryonic mortality (Rafferty *et al.* 2011). This indicates that embryos may fail to break from pre-ovipositional arrest after oviposition if arrest is prolonged. Leatherback turtles generally experience low hatching success rates and are a species of conservation concern (Eckert *et al.* 2012; Wallace *et al.* 2013). Therefore it seems pertinent to further investigate the role that pre-ovipositional arrest plays in development of leatherback turtle eggs with experimental investigation both 'in-situ' and 'ex-situ'. Furthermore, since we know that increased oxygen is the trigger that breaks embryos from pre-ovipositional arrest, and it appears embryos may be failing to break this arrest, it is worthwhile investigating whether increasing oxygen concentration above normoxic levels (hyperoxia) has any positive impact upon development and subsequent hatching success of leatherback turtles.

There may also be an important role played by pre-ovipositional embryonic arrest in the evolution of an interesting behavioural reproductive polymorphism exhibited in the ridley (*Lepidochelys*) genus. Ridley turtles either nest solitarily as other species of sea turtle do, or nest in synchronised mass nesting events termed arribadas (Bernardo and Plotkin 2007). The inter-nesting interval (period between subsequent clutches) varies depending on the nesting tactic employed; 14 days for solitary nesters compared to 28 days for arribada nesters (Kalb 1999).

This suggests that eggs spend varied periods of time in pre-ovipositional embryonic arrest, depending on the reproductive tactic used by the mother. Further investigation of the role pre-ovipositional embryonic arrest plays in the evolution and maintenance of this unique life-history trait is warranted.

1.4 Study aims and thesis structure

Given the importance of pre-ovipositional arrest for the ecology and evolution of turtles, and possibly crocodilians, the overarching aim of the studies described in my thesis is to further investigate the intricacies of how pre-ovipositional embryonic arrest functions and what management and conservation implications it may have. In order to address this aim I designed a series of experiments (Chapters 2-6) to address a range of specific questions regarding pre-ovipositional embryonic arrest in crocodilians and turtles (Figure 1.1).

The study described in Chapter 2 was conducted at Heron Island, Australia, using green sea turtles (*Chelonia mydas*) as a model species with the aim to assess how long after oviposition turtle embryos require in normoxia before they break from pre-ovipositional embryonic arrest and if they can subsequently re-enter arrest. Chapter 2 has already been published in the journal Physiological and Biochemical Zoology (Williamson *et al.* 2017a). This study provided important information about when embryos should no longer be moved or returned to hypoxia.

Previous experimental studies of extension of pre-ovipositional embryonic arrest in sea turtles have used expensive and cumbersome Perspex (clear polymethyl methacrylate plastic, also called Plexiglass or Lucite) chambers to create a hypoxic incubation environment. The aim of the studies described in Chapter 3 was to assess whether an efficient and cost-effective method can be developed to extend arrest and if extending pre-ovipositional embryonic arrest protects eggs

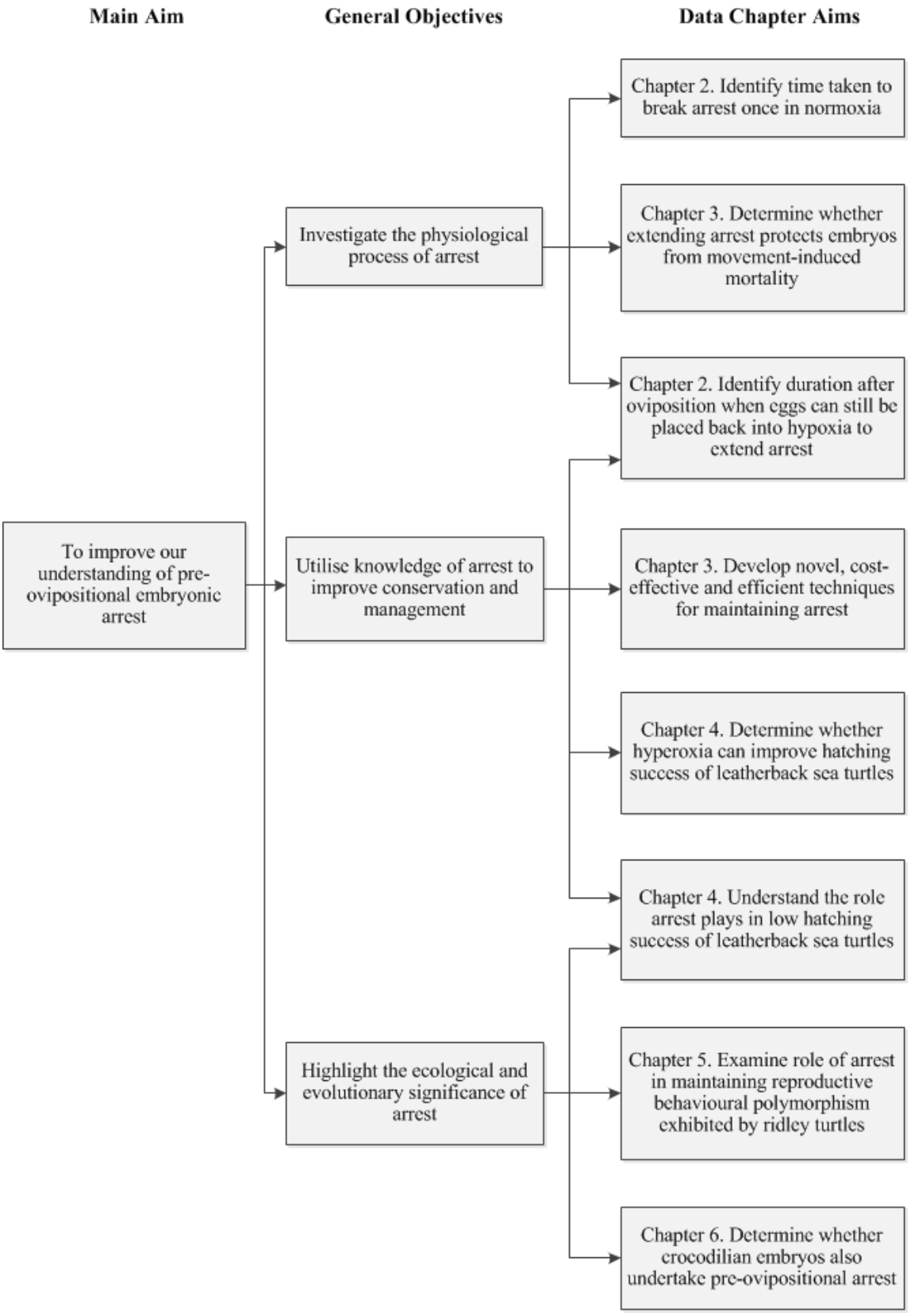


Figure 1.1 Research aims and general structure for thesis.

from movement-induced mortality. This was undertaken using eggs of olive ridley sea turtles (*Lepidochelys olivacea*) collected from Ostional, Costa Rica. Chapter 3 has been published in the journal Biological Conservation (Williamson *et al.* 2017b). These studies provided important information for conservationists and managers regarding the potential for pre-ovipositional embryonic arrest as a tool to improve outcomes when translocating nests.

The study described in Chapter 4 was conducted at Pacuare Nature Reserve on the Caribbean coast of Costa Rica. The primary aim of this study was to assess the efficacy of using hyperoxia during incubation to reduce early-stage embryonic mortality in leatherback turtles (*Dermochelys coriacea*). A secondary aim was to assess whether extended pre-ovipositional arrest through hypoxic incubation negatively impacts embryonic development. The findings of this chapter contribute novel information on the reproductive biology of this species of global conservation concern.

The study described in Chapter 5 was designed to determine whether olive ridley turtles (*L. olivacea*) eggs oviposited during an arribada differ from those oviposited during solitary nesting events in their capacity to extend pre-ovipositional embryonic arrest after oviposition. This study was completed using eggs collected from Ostional, Costa Rica. The findings contribute to our understanding to how this interesting and unique behavioural reproductive polymorphism functions, improving our ecological and evolutionary understanding of the arribadas.

The study described in Chapter 6 was aimed at elucidating whether crocodilians also arrest embryonic development prior to oviposition. It was conducted at Crocodylus Park (Darwin, Australia) on captive saltwater crocodiles (*Crocodylus porosus*). Chapter 6 is in press in the journal Royal Society Open Science (Williamson *et al.* 2017c). The findings from this study

indicate that hypoxia-induced pre-ovipositional embryonic arrest does not occur in saltwater crocodiles, which advances our understanding of the evolution of pre-ovipositional embryonic arrest within reptilians.

Chapter 7 is a general discussion and integration of the results from all five experiments. The general trends and differences found across the various experiments are discussed. The ecological and evolutionary implications of the findings from these experiments are also highlighted along with suggestions for future research. Finally, this chapter includes a conclusion for the thesis.

1.5 References

Bernardo, J, Plotkin, PT (2007) An evolutionary perspective on the arribada phenomenon and reproductive behavioral polymorphism of olive ridley sea turtles (*Lepidochelys olivacea*). In 'Biology and conservation of ridley sea turtles.' (Ed. PT Plotkin.) pp. 58-87. (The John Hopkins University Press: Baltimore, Maryland)

Birkhead, TR, Møller, AP (1993) Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society* **50**, 295-311.

Bjorndal, KA, Carr, A (1989) Variation in clutch size and egg size in the green turtle nesting population at Tortuguero, Costa Rica. *Herpetologica* **45**, 181-189.

Blackburn, DG (1998) Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. *Journal of Experimental Zoology* **282**, 560-617.

Blackburn, DG (2006) Squamate reptiles as model organisms for the evolution of viviparity. *Herpetological Monographs* **20**, 131-146.

Buhlmann, K, Lynch, T, Whitfield Gibbons, J, Greene, J (1995) Prolonged egg retention in the turtle *Deirochelys reticularia* in South Carolina. *Herpetologica* **51**, 457-462.

Castanet, J (1994) Age estimation and longevity in reptiles. *Gerontology* **40**, 174-192.

Deeming, CD (1991) Reasons for the dichotomy in egg turning in birds and reptiles. In 'Egg incubation: Its effects on embryonic development in birds and reptiles.' (Eds CD Deeming, MW Ferguson.) pp. 307-324. (Cambridge University Press: Cambridge, UK)

Duellman, WE, Trueb, L (1986) 'Biology of amphibians.' (John Hopkins University Press: Baltimore, Maryland)

Eckert, KL, Wallace, BP, Frazier, JG, Eckert, SA, Pritchard, PCH, 2012. Synopsis of the biological data on the leatherback sea turtle (*Dermochelys coriacea*). U.S. Department of Interior, Fish and Wildlife Service, Washington, D.C.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Ewert, MA (1991) Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In 'Egg incubation: ifs effects on embryonic development in birds and reptiles.' (Eds DC Deeming, MWJ Ferguson.) pp. 173-191. (Cambridge University Press: Cambridge, UK)

Ewert, MA, Wilson, DS (1996) Seasonal variation of embryonic diapause in the striped mud turtle (*Kinosternon baurii*) and general considerations for conservation planning. *Chelonian Conservation and Biology* **2**, 43-54.

Ferguson, MWJ (1982) The structure and composition of the eggshell and embryonic membranes of *Alligator mississippiensis*. *The Transactions of the Zoological Society of London* **36**, 99-152.

Ferguson, MWJ (1985) Reproductive biology and embryology of the crocodilians. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 329-491. (John Wiley & Sons: New York)

Fordham, D, Georges, A, Corey, B (2006) Compensation for inundation-induced embryonic diapause in a freshwater turtle: Achieving predictability in the face of environmental stochasticity. *Functional Ecology* **20**, 670-677.

Fordham, DA, Georges, A, Corey, B (2007) Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise. *Aquaculture* **270**, 105-114.

Hirth, HF (1980) Some aspects of the nesting behavior and reproductive biology of sea turtles. *American Zoologist* **20**, 507-523.

Janzen, FJ, Tucker, JK, Paukstis, GL (2000) Experimental analysis of an early life-history stage: Selection on size of hatchling turtles. *Ecology* **81**, 2290-2304.

Kalb, HJ (1999) Behavior and physiology of solitary and arribada nesting olive ridley sea turtles (*Lepidochelys olivacea*) during the internesting period. Texas A&M University.

Kennett, R, Georges, A, Palmerallen, M (1993) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Kohring, RR (1995) Reflections on the origin of the amniote egg in the light of reproductive strategies and shell structure. *Historical Biology* **10**, 259-275.

Limpus, CJ, Baker, V, Miller, JD (1979) Movement induced mortality of loggerhead eggs. *Herpetologica* **35**, 335-338.

Little, C (1990) 'The terrestrial invasion: An ecophysiological approach to the origins of land animals.' (Cambridge University Press: Cambridge)

Lynn, WG, von Brand, T (1945) Studies on the oxygen consumption and water metabolism of turtle embryos. *The Biological Bulletin* **88**, 112-125.

McIlhenny, EA (1934) Notes on incubation and growth of alligators. *Copeia* **1934**, 80-88.

Miller, JD (1982) Development of marine turtles. University of New England.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Miller, JD (1997) Reproduction in sea turtles. In 'The biology of sea turtles.' (Eds PL Lutz, JA Musick.) (CRC Press: Boca Raton, Florida)

Miller, JD, Limpus, CJ (1983) A method for reducing movement-induced mortality in turtle eggs. *Marine Turtle Newsletter* 10-11.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Reina, RD (2014) The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. *Journal of Experimental Marine Biology and Ecology* **450**, 91-97.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**,

Reese, AM (1931) The ways of the alligator. *The Scientific Monthly* **33**, 321-335.

Reisz, RR (1997) The origin and early evolutionary history of amniotes. *Trends in Ecology & Evolution* **12**, 218-222.

Renfree, MB, Shaw, G (2000) Diapause. *Annual Review of Physiology* **62**, 353-375.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Risley, P (1944) Arrested development in turtle embryos. *The Anatomical Record* **88**, 454-455.

Shine, R (1985) The evolution of viviparity in reptiles: an ecological analysis. In 'Biology of the Reptilia.' (Eds C Gans, F Billett.) Vol. 15 pp. 605-694. (John Wiley and Sons: New York)

Shine, R (1988) Parental care in reptiles. In 'Biology of the Reptilia.' (Eds C Gans, RB Huey.) Vol. 16 pp. 275-329. (John Wiley and Sons: New York)

Shine, R (2005) Life-history evolution in reptiles. *Annual Review of Ecology, Evolution, and Systematics* **36**, 23-46.

Shine, R, Iverson, JB (1995) Patterns of survival, growth and maturation in turtles. *Oikos* **72**, 343-348.

Somaweera, R, Brien, M, Shine, R (2013) The role of predation in shaping crocodilian natural history. *Herpetological Monographs* **27**, 23-51.

Thorbjarnarson, JB (1996) Reproductive characteristics of the order *Crocodylia*. *Herpetologica* **52**, 8-24.

Wake, MH (1993) Evolution of oviductal gestation in amphibians. *Journal of Experimental Zoology* **266**, 394-413.

Wallace, BP, Tiwari, M, Girondot, M (2013) *Dermochelys coriacea*. *The IUCN Red List of Threatened Species* **2013**

Williamson, SA, Evans, RG, Reina, RD (2017a) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Williamson, SA, Evans, RG, Robinson, NJ, Reina, RD (2017b) Hypoxia as a novel method for preventing movement-induced moratlity during translocation of turtle eggs. *Biological Conservation* **216**, 86-92.

Williamson, SA, Evans, RG, Manolis, SC, Webb, GJ, Reina, RD (2017c) Ecological and evolutionary significance of a lack of capacity for extended developmental arrest in crocodilian eggs. *Royal Society Open Science* **4**, 171439.

Chapter 2. When is embryonic arrest broken in turtle eggs?

Sean A. Williamson[1], Roger G. Evans[2], and Richard D. Reina[1]

[1] School of Biological Sciences, Monash University

[2] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University

Published under the same name in *Physiological and Biochemical Zoology* (see Appendix A).

2.1 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 hours and 20 days 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 days, commencing 30 minutes to 48 hours 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 hours or less, hatching success did not differ from a control group. If the hypoxic treatment began after 16 hours 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 hours of oviposition), it is possible to extend embryonic arrest for at least three days 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, Pre-ovipositional Arrest, Reproduction, Oxygen

2.2 Introduction

Turtle ecology and evolutionary history is greatly influenced by a single physiological trait; pre-ovipositional 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 enter pre-ovipositional 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).

Pre-ovipositional 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 favourable 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 hours 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 pre-ovipositional 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 pre-ovipositional 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 due to 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 pre-ovipositional 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 to break pre-ovipositional 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 prior to 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.* 1987b) 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.* 1987b). 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 hours to 20 days of development, these fused membranes can easily rupture, resulting in the death of the embryo (Limpus *et al.* 1979; Webb *et al.* 1987a; Webb *et al.* 1987b). 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). As the increase in the partial pressure of oxygen at the time of laying is the trigger that breaks pre-ovipositional 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 minutes 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 C.*

longicollis) when eggs where laid underwater and kept submerged. Knowing the duration of exposure to normoxia required to break pre-ovipositional 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 upon 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 pre-ovipositional arrest. Kennett *et al.* (1993) was unable to observe re-entry of *C. rugosa and C. longicollis* eggs into arrest at 10 and 20 days after oviposition, but we might expect that embryonic development was relatively advanced at that point, likely between Yntema's (1968) stage 9 and 20 of development (Ewert 1985). It seems possible that re-entry into hypoxia may be possible at a much earlier stage. Any ability of embryos to re-enter 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 unfavourable environmental conditions such as nest inundation by heavy rains or extreme tidal events. We hypothesised that the breaking of arrest occurs approximately 12 hours 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 pre-ovipositional 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 pre-ovipositional 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 re-enter 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.

2.3 Methods

2.3.1 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).

2.3.2 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 the 29th of January, 2015, between 20:00 and 23:00, 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 the 3rd of February, 2015, between 19:00 and 22:00. 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 was measured once the eggs arrived at the laboratory.

2.3.3 Hypoxic treatments to assess embryonic arrest

Eggs from each clutch were randomly allocated to one of ten 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, see below) and then placed into hypoxia (~1% O_2 in a Perspex chamber, see below) at 0.5, 1, 2, 4, 8, 12, 16, 24 and 48 hours post-oviposition respectively (n = 36 in each case). These eggs remained under hypoxic conditions for three days before being incubated in normoxia again. For example, eggs in the 0.5 h group were in normoxia for 30 minutes after oviposition, then in hypoxia until 3 days and 30 minutes after oviposition, then in normoxia until hatching, while the 1 h group were in normoxia for 1 h after oviposition, then hypoxia until 3 days and 1 h after oviposition, then in normoxia for the rest of development, and so on for the other groups.

Eggs were maintained in hypoxia by placing them into airtight Perspex containers (Resi-Plex Plastics, Vic, Australia) 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 in-flow and out-flow valve at opposite ends of the

box and industrial grade 100% nitrogen gas (BOC, North Ryde, Australia) 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 (PO_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 (PO_2 ~8 mmHg). The containers were then sealed and placed in incubators (GQF HovaBator model 1632; Grandview Management, Baldivis, Australia) set to 28°C. The containers were re-gassed approximately every 24 hours over the three-day treatment period. During re-gassing the PO_2 was monitored as the gas flowed through the chamber to ensure the PO_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.

2.3.4 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 callipers to measure the

maximum diameter of the white spot to the nearest mm. Eggs that formed a white spot prior to being placed into hypoxia did not have their white spot growth measured whilst 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 prior to the egg being placed in hypoxia and immediately following removal of the egg from hypoxia. Any eggs that showed visible signs of embryonic death (such as green discolouration 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 days and 9 days post-oviposition respectively. Prior to 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.

2.3.5 Excavation of nests

Approximately two days 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 classifications of the stage of development as described in detail by Rafferty *et al.* (2011).

2.3.6 Statistical analysis

Analysis of variance (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 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 tests with Bonferroni corrections for pair-wise 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 Cochran-Mantel-Haenszel tests were conducted using Chi-squared tests with Bonferroni corrections for pairwise comparisons with the independent variable of interest being the treatment group. A chi-squared 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 days 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 prior to 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. Cochran-Mantel-Haenszel test with Bonferroni corrections for pair-wise 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 treatments groups). Post hoc analysis of the Cochran-Mantel-Haenszel tests was conducted using Chi-squared tests with Bonferroni corrections for pairwise comparisons for the independent variable of treatment group. All analyses were performed using R software (R Core Team 2013). All values are mean ± standard error unless otherwise stated. Two-tailed $P \leq 0.05$ was considered statistically significant.

2.4 Results

2.4.1 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 2.1). 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 whilst in hypoxia. There was no variation in the aerobic time taken to form a white spot ($F_{9, 43} = 1.50$, $p = 0.18$; Figure 2.1). The latency to white

spot formation across all groups was 39 ± 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 (Figure 2.1). 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 hours or less. There was also systematic variation in the proportion of eggs in each treatment that formed white spots prior to hypoxia ($X^2_{CMH} = 15.73$, d.f. = 5, $p < 0.01$; Figure 2.1). That is, no eggs formed white spots prior to hypoxia when the delay before hypoxic incubation was 16 hours or less. In contrast, 47% formed white spots prior to hypoxia when the delay was 24 hours, and 97% formed white spots prior to hypoxia when the delay was 48 hours.

Growth rate of the white spot (mm/h) was affected by exposure to hypoxia ($F_{9, 41} = 10.46$, $p < 0.0001$, Figure 2.2). 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.

Table 2.1. Average mass and diameter of green turtle eggs from each treatment.

	Control	0.5h	1h	2h	4h	8h	12h	16h	24h	48h
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

Eggs were placed into hypoxia for three days at a different time point after oviposition ranging from 0.5 to 48 hours (n = 32-40, N = 355). Data are mean ± standard error.

There was no detectable development of embryos whilst in hypoxia, as ascertained from opening and staging eggs from each treatment before and after hypoxia (Table 2.2). 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 2.2). There was no detectable growth of embryos until after 12 hours 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 2.2).

Table 2.2. Stage of embryonic development of eggs randomly selected from each treatment.

	Control	0.5h	1h	2h	4h	8h	12h	16h	24h	48h
Before hypoxia	6 & 6	6 & 6	6 & 6	6 & 6	6 & 6	6 & 6	7 & 6	7 & 7	8 & 8	10 & 9
After hypoxia	N/A	6 & 6	6 & 6	6 & 6	6 & 6	6 & 6	6 & 7	7 & 7	8 & 8	9 & 9

Embryos were staged according to Miller's (1985) 31-stage developmental chronology. Eggs were placed into hypoxia for three days at a different time point after oviposition ranging from 0.5 to 48 hours. Eggs (n = 2) were opened at the time of collection (control), before and after each treatment group was placed into hypoxia (N = 38).

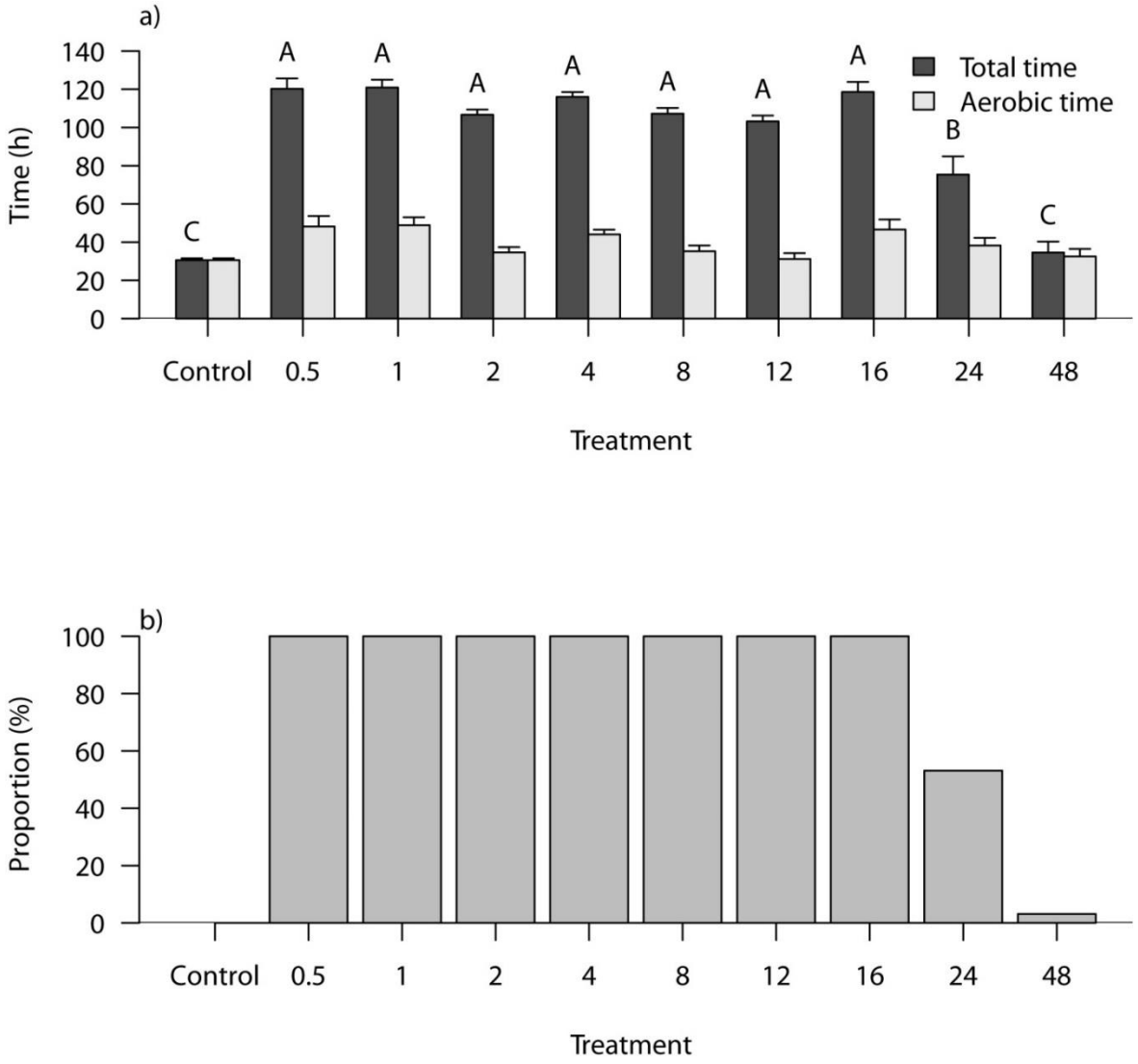


Figure 2.1. a) Latency after oviposition until formation of the white spot on eggs and b) proportion of white spots to form after hypoxia in each treatment group. Eggs were placed into hypoxia for three days at various time-points after oviposition ranging, from 0.5 – 48 hours. Data for a) are mean ± SE of n = 31-37 per group. In a) dark grey bars represent total time and light grey bars represent aerobic time (total time excluding time in hypoxia). In b) the 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; $p > 0.05$). When the letters above the error bars for total time are the same, the latency to white spot formation did not differ significantly between the corresponding treatment groups (ANOVA and Tukey's HSD tests; $p < 0.05$).

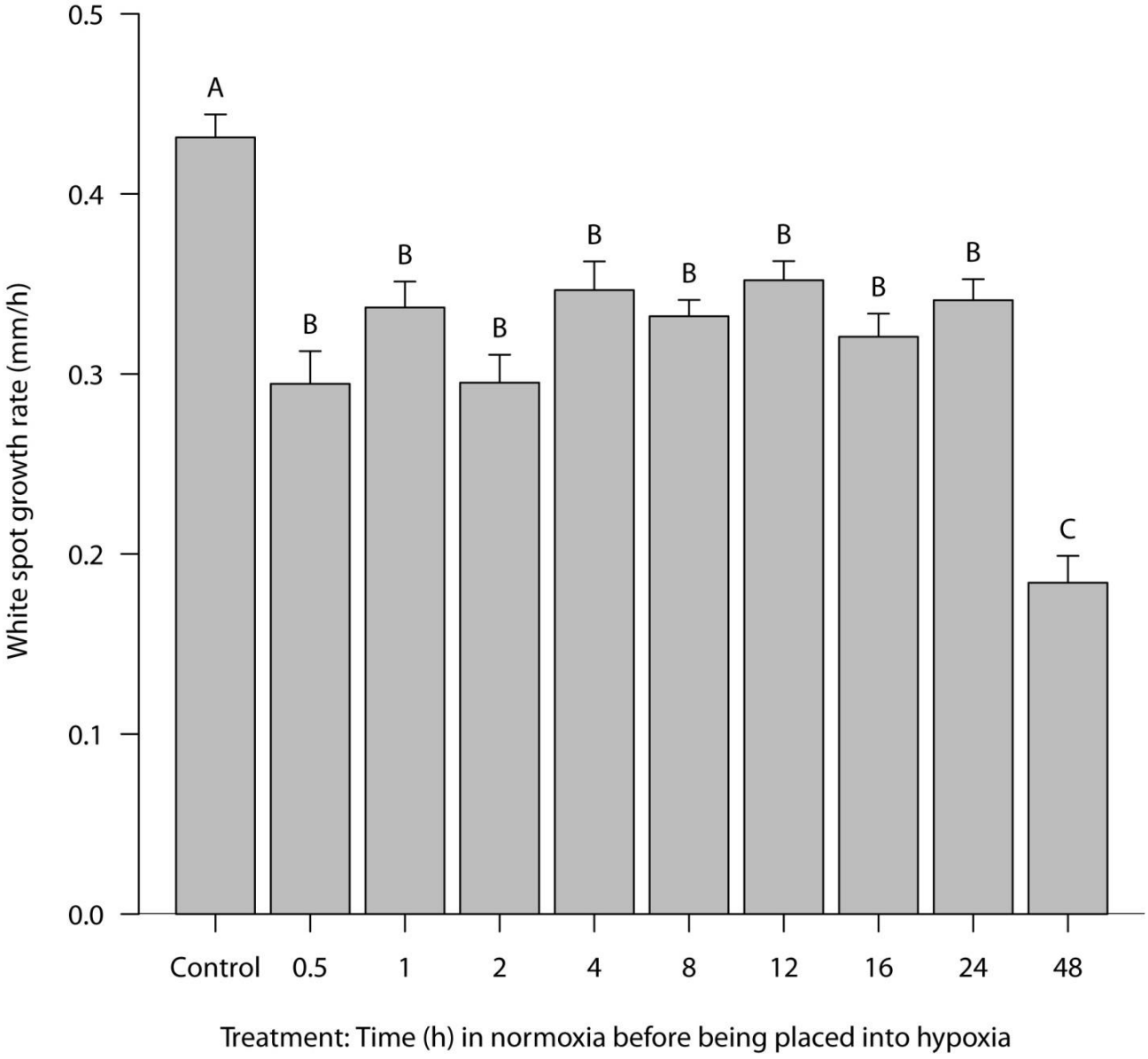


Figure 2.2. Rate of growth of the white spot on eggs in each treatment group after eggs were removed from hypoxia. Eggs were placed into hypoxia for three days at various time-points after oviposition ranging, from 0.5 – 48 hours. Data are mean ± SE of n = 25-37. When letters above each bar are the same, the white spot growth rate did not differ significantly between the corresponding treatment groups (ANOVA and Tukey's HSD test; $p < 0.05$).

2.4.2 Hatching success

There was significant variation in hatching success between the treatments amongst the different clutches (X^2_{CMH} = 130.02, d.f. = 5, p < 0.0001).The difference between clutches was due to the significant difference between the hatching success depending on the night of collection (X^2 = 97.66, d.f. = 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 to 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 treatment-group variation in hatching success was statistically significant (X^2 = 107.20, d.f. = 9, p < 0.0001, Figure 2.3). Within the eggs from the first collection, hatching success was similar in the 0.5, 1, 2, 4, 8, and 12 h treatments compared to control, while none of the eggs in the 16, 24 or 48 h treatment-groups hatched (Figure 2.3).

2.4.3 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) (X^2_{CMH} = 6.38, d.f. = 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 (Figure 2.4). 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's (1996) Stage 0; no embryo or blood vessels visible).

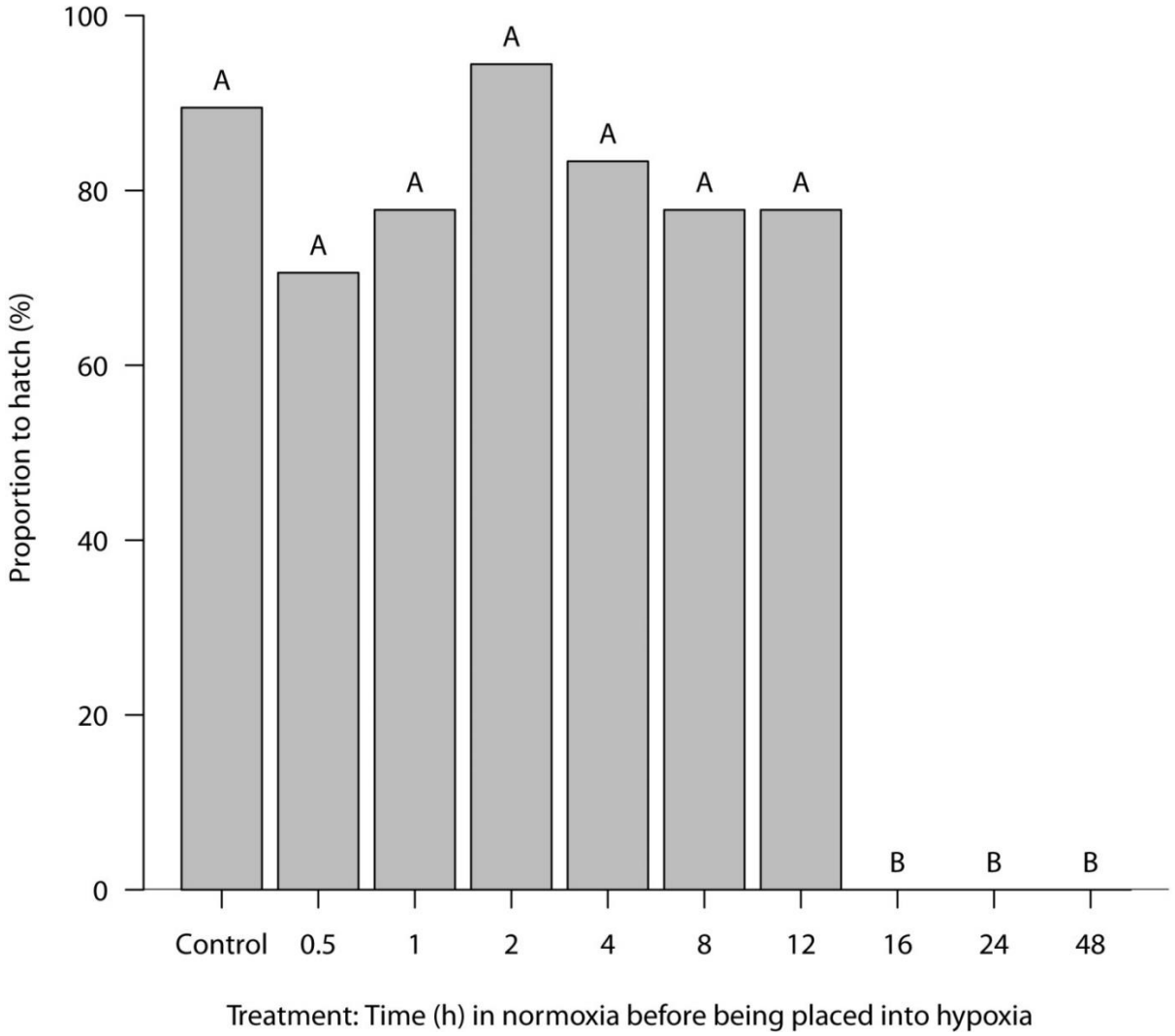


Figure 2.3. Hatching success, proportion of eggs to hatch, in each treatment where eggs were placed into hypoxia for 3 days after a differing amount of time in normoxia (n = 16-20). Eggs were placed into hypoxia for three days at various time-points after oviposition, ranging from 0.5 – 48 hours. When letters above each bar are the same, the hatching success did not differ significantly between the corresponding treatment groups (Bonferroni corrected Chi-squared test; $p < 0.05$).

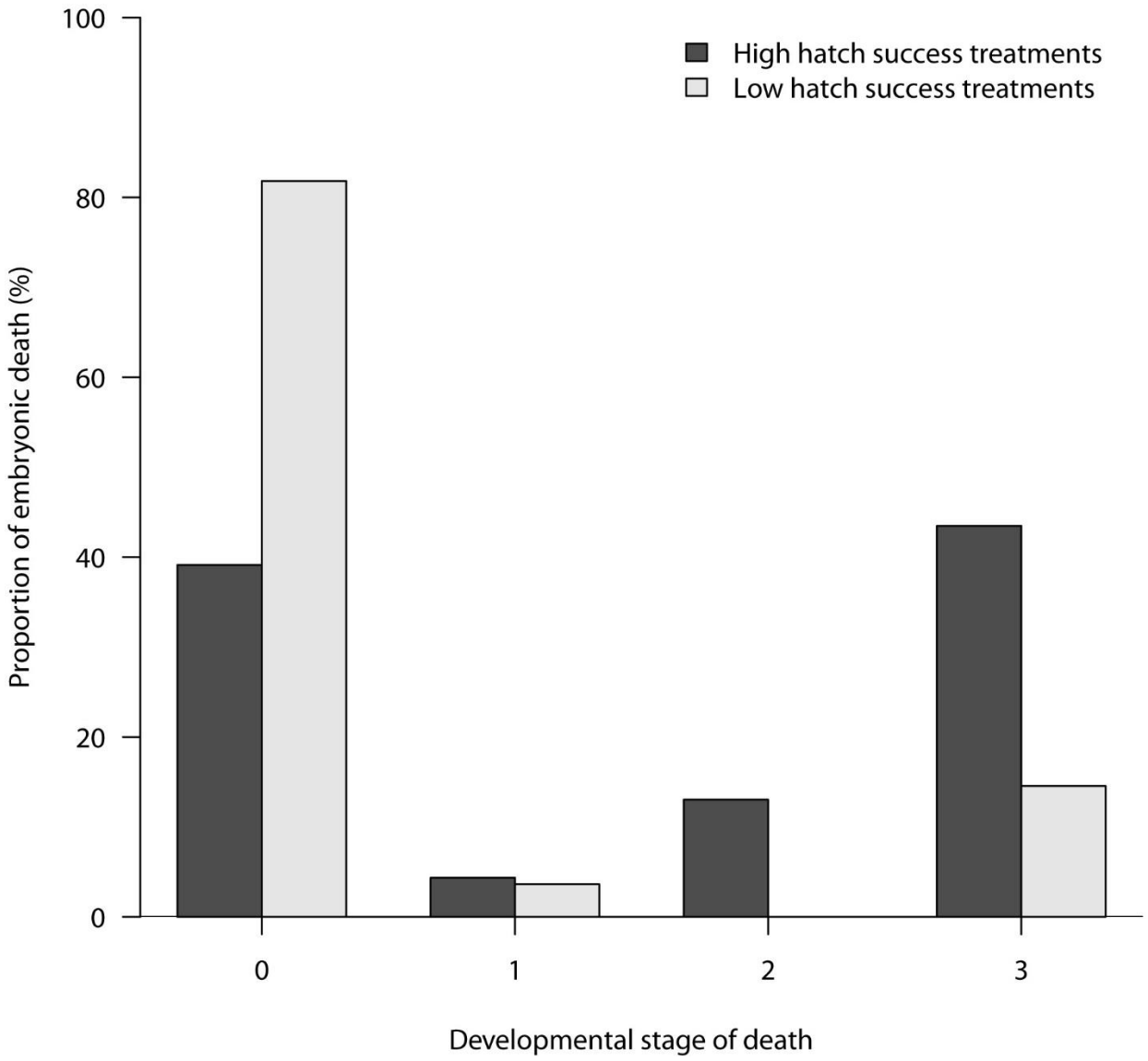


Figure 2.4. Proportion of embryos from the first collection night that died at each developmental stage. Eggs were placed into hypoxia for three days at various time-points after oviposition, ranging from 0.5 – 48 hours. Treatments with high hatching success (control and 0.5 – 12 h treatments) are represented by the dark grey bars (n = 23) and the treatments with low hatching success (16 – 48 h treatments) are represented by the light grey bars (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 0-3.

2.5 Discussion

We found that after more than 12 hours 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 hours or less, eggs survived a subsequent three day 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 pre-ovipositional embryonic arrest. This is further supported by the absence of detectable embryonic development during the three-day period of hypoxic incubation, while control eggs in normoxia continued to develop. Overall our results show that pre-ovipositional arrest is broken within 12 to 16 hours 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 hours, the embryos would no longer be capable of protecting themselves by pausing oxygen consumption and embryonic development. Our findings build upon observations from multiple species of marine and freshwater turtles that the first discernible sign of post-ovipositional development occurs between 12 to 24 hours 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 pre-ovipositional 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 to 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). 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. Booth pers. comm., C. Cavallo pers. comm., B. Bentley pers. comm.). 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.* 1983a; Webb *et al.* 1983b). 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.* 1987b). 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 extended pre-ovipositional 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 utilise 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 Jr 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 pre-ovipositional embryonic arrest. This could then result in the embryonic and egg shell membranes fusing whilst the egg is still within the mother. Consequently, embryos would then be susceptible to mortality either from exaggerated movements of the mother whilst 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 they either 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 'road-block' to the evolution of viviparity, imposed by the nature of hypoxic embryonic arrest, could only be overcome 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 upon 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, as 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 pre-ovipositional embryonic arrest is warranted (Rafferty and Reina 2012). In turtles, a physiological constraint of low oxygen in the oviduct is utilised as an adaptation 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 where 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 to 11 days of development depending on whether or not they were placed into hypoxia for 3 days or not. However embryos from the second collection would have been between 9 to 6 days of

development when they were moved. As 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 minimising movement during egg-burial, it appears that their sensitivity to movement was too great. Our embryonic staging data also support this hypothesis, as 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 neighbouring eggs of different treatments would have been controlled for.

In conclusion, our findings provide the first experimental evidence that pre-ovipositional embryonic arrest is broken between 12 and 16 hours after laying in green turtle eggs. We have also shown it is possible to place eggs back into hypoxia before this time has elapsed, in order to extend pre-ovipositional arrest and delay development, with no negative impact upon hatching success. However, developmental arrest is broken at a time-point between 12 to 16 hours after oviposition. Consequently, embryos die if they are then incubated in hypoxia for a substantial period. This information provides further evidence that eggs should only be relocated or moved under normoxic conditions within 12 hours 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 pre-ovipositional 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.

Pre-ovipositional embryonic arrest provides protection from movement-induced mortality. However, this adaptation also represents a road-block to evolution of viviparity, since even slight increases in the stage of embryonic development prior to 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.

2.6 Acknowledgements

We thank B. Holsworth and the Holsworth Wildlife Research Endowment for generous financial support. Financial support to S.W. was made possible through an Australian Postgraduate Award from the Australian Research Council. Thank you to J. Guy from Good Food Solutions and his family for generously catering for our stay on the island. Thanks also to C. Rings, T. Rafferty, and C. Rings for their support during the project. We are grateful to the assistance D. Annese, E. Perkins and M. Roberts at the Heron Island Research Station provided us whilst completing the study. Thanks to J. Ferguson and the Queensland National Parks Turtle Volunteers for their assistance in the field. Finally a heartfelt thank you in particular to C. Neilson for continual support and volunteering over multiple weeks to help with data collection at all hours of the night.

2.7 References

Andrews, RM (2002) Low oxygen: A constraint on the evolution of viviparity in reptiles. *Physiological and Biochemical Zoology* **75**, 145-154.

Andrews, RM, Mathies, T (2000) Natural history of reptilian development: Constraints on the evolution of viviparity. *BioScience* **50**, 227-238.

Aubret, F, Blanvillain, G, Kok, PJR (2015) Myth busting? Effects of embryo positioning and egg turning on hatching success in the water snake *Natrix maura*. *Scientific Reports* **5**, 13385.

Beggs, K, Young, J, Georges, A, West, P (2000) Ageing the eggs and embryos of the pig-nosed turtle, *Carettochelys insculpta* (*Chelonia: Carettochelydidae*), from northern Australia. *Canadian Journal of Zoology* **78**, 373-392.

Booth, DT (2000) Incubation of eggs of the Australian broad-shelled turtle, *Chelodina expansa* (*Testudinata: Chelidae*), at different temperatures: Effects on pattern of oxygen consumption and hatchling morphology. *Australian Journal of Zoology* **48**, 369-378.

Buhlmann, K, Lynch, T, Whitfield Gibbons, J, Greene, J (1995) Prolonged egg retention in the turtle *Deirochelys reticularia* in South Carolina. *Herpetologica* **51**, 457-462.

Deeming, CD (1991) Reasons for the dichotomy in egg turning in birds and reptiles. In 'Egg incubation: Its effects on embryonic development in birds and reptiles.' (Eds CD Deeming, MW Ferguson.) pp. 307-324. (Cambridge University Press: Cambridge, UK)

Deeming, CD, Thompson, MB (1991) Gas exchange across reptilian eggshells. In 'Egg incubation: Its effects on embryonic development in birds and reptiles.' (Eds CD Deeming, MW Ferguson.) pp. 277-284. (Cambridge University Press: Cambridge, UK)

Ewert, MA (1985) Embryology of Turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Ewert, MA (1991) Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In 'Egg incubation: ifs effects on embryonic development in birds and reptiles.' (Eds DC Deeming, MWJ Ferguson.) pp. 173-191. (Cambridge University Press: Cambridge, UK)

Kennett, R, Georges, A, Palmerallen, M (1993) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Leslie, AJ, Penick, DN, Spotila, JR, Paladino, FV (1996) Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990-1991. *Chelonian Conservation & Biology* **2**, 159-168.

Licht, P (1980) Evolutionary and functional aspects of pituitary gonadotropins in the green turtle, *Chelonia mydas*. *American Zoologist* **20**, 565-574.

Licht, P (1982) Endocrine patterns in the reproductive cycle of turtles. *Herpetologica* **38**, 51-61.

Licht, P, Owens, DW, Cliffton, K, Penaflores, C (1982) Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, *Lepidochelys olivacea*. *General and Comparative Endocrinology* **48**, 247-253.

Limpus, CJ, Baker, V, Miller, JD (1979) Movement induced mortality of loggerhead eggs. *Herpetologica* **35**, 335-338.

Marcellini, DL, Davis, SW (1982) Effects of handling on reptile egg hatching. *Herpetological Review* **13**, 43-44.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Miller, JD, Limpus, CJ (1983) A method for reducing movement-induced mortality in turtle eggs. *Marine Turtle Newsletter* 10-11.

Owens, D, Morris, YA (1985) The comparative endocrinology of sea turtles. *Copeia* **1985**, 723-735.

Parker, SL, Andrews, Robin M (2006) Evolution of viviparity in sceloporine lizards: In utero PO_2 as a developmental constraint during egg retention. *Physiological and Biochemical Zoology* **79**, 581-592.

Parker, SL, Murphy, CR, Thompson, MB (2010) Uterine angiogenesis in squamate reptiles: Implications for the evolution of viviparity. *Herpetological Conservation and Biology* **5**, 330-334.

Parmenter, CJ (1980) Incubation of the eggs of the green sea turtle, *Chelonia mydas*, in Torres Strait, Australia: The effect of movement on hatchability. *Australian Wildlife Research* **7**, 487-491.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

R Core Team (2013) R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna, Austria.

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**,

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Rusli, MU, Booth, DT, Joseph, J (2016) Synchronous activity lowers the energetic cost of nest escape for sea turtle hatchlings. *Journal of Experimental Biology* **219**, 1505-1513.

Santos, RG, Pinheiro, HT, Martins, AS, Riul, P, Bruno, SC, Janzen, FJ, Ioannou, CC (2016) The anti-predator role of within-nest emergence synchrony in sea turtle hatchlings. *Proceedings of the Royal Society B: Biological Sciences* **283**, 20160697.

Shine, R (1985) The evolution of viviparity in reptiles: an ecological analysis. In 'Biology of the Reptilia.' (Eds C Gans, F Billett.) Vol. 15 pp. 605-694. (John Wiley and Sons: New York)

Shine, R, Guillette Jr, LJ (1988) The evolution of viviparity in reptiles: A physiological model and its ecological consequences. *Journal of Theoretical Biology* **132**, 43-50.

Spencer, R-J, Thompson, MB, Banks, PB (2001) Hatch or wait? A dilemma in reptilian incubation. *Oikos* **93**, 401-406.

Thompson, MB (1985) Functional significance of the opaque white patch in eggs of *Emydura macquarii*. In 'Biology of Australasian frogs and reptiles.' (Eds G Grigg, R Shine, H Ehmann.) pp. 387-395. (Surrey Beatty in association with The Royal Zoological Society of New South Wales: Chipping Norton, NSW)

Thompson, MB (1993) Oxygen consumption and energetics of development in eggs of the leatherback turtle, *Dermochelys coriacea*. *Comparative Biochemistry and Physiology - A Physiology* **104**, 449-453.

Warner, DA, Moody, MA, Telemeco, RS (2011) Is water uptake by reptilian eggs regulated by physiological processes of embryos or a passive hydraulic response to developmental

environments? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **160**, 421-425.

Webb, GJW, Manolis, CS, Dempsey, KE, Whitehead, PJ (1987a) Crocodilian eggs: A function overview. In 'Wildlife Management; Crocodiles and Alligators.' (Eds GJW Webb, CS Manolis, PJ Whitehead.) pp. 417-422. (Surrey Beatty: Chipping Norton, Australia)

Webb, GJW, Manolis, SC, Buckworth, R (1983a) *Crocodylus johnstoni* in the McKinlay River Area N. T, VI.* Nesting Biology. *Wildlife Research* **10**, 607-637.

Webb, GJW, Manolis, SC, Buckworth, R, Sack, GC (1983b) An examination of *Crocodylus porosus* nests in two northern Australian freshwater swamps, with an analysis of embryo mortality. *Wildlife Research* **10**, 571-605.

Webb, GJW, Manolis, SC, Whitehead, PJ, Dempsey, K (1987b) The possible relationship between embryo orientation opaque banding and the dehydration of albumen in crocodile eggs. *Copeia* **1987**, 252-257.

Yntema, CL (1968) A series of stages in the embryonic development of *Chelydra serpentina*. *Journal of Morphology* **125**, 219-251.

Chapter 3. Hypoxia as a novel method for preventing movement-induced mortality during translocation of turtle eggs.

Sean A. Williamson[1], Roger G. Evans[2], Nathan J. Robinson[3, 4, 5] and Richard D. Reina[1]

[1] School of Biological Sciences, Monash University, Clayton VIC 3800, Australia

[2] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton VIC 3800, Australia

[3] The Leatherback Trust, Goldring-Gund Marine Biology Station, Playa Grande, Costa Rica

[4] Department of Biology, Indiana-Purdue University, Fort Wayne, Indiana, USA

[5] Present address: Cape Eleuthera Institute, Eleuthera, The Bahamas

This chapter was published under the same name in *Biological Conservation* (see Appendix B).

3.1 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.

Keywords: *Embryonic Development, Turtle Conservation, Hypoxia, Egg Relocation, Embryonic Arrest, Olive Ridley*

3.2 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 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 hours 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; Fordham *et al.* 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*).

3.3 Materials and methods

3.3.1 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).

3.3.2 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 minutes.

3.3.3 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

Figure 3.1. 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.

"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; 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-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} (Figure 3.1). To ensure each vessel had reached approximately 1% oxygen v/v (PO_2 ~8 mmHg) 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

(Figure 3.1). 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 hours, 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 degrees 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.

3.3.4 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.

3.3.5 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) x 100

3.3.6 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 two minutes that individual trial was abandoned and the hatchling was given a score of two minutes. The hatchling was then allowed a five-minute break before it was tested again.

3.3.7 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).

3.4 Results

3.4.1 Egg development and white spot formation

There was no significant difference between treatments in the proportion of eggs to form white spots ($X^2_{CMH} = 0.69$, d.f. = 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 ($H = 232.76$, d.f. = 3, $p < 0.0001$). Eggs in the Perspex and ziplock treatments took approximately three days and six hours 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 (Figure 3.2a). Eggs in the vacuum treatment took two days and nine hours longer than the control to form white spots (Figure 3.2a). 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 ($H = 151.66$, d.f. = 3, $p < 0.0001$ Figure 3.2b). The Perspex and ziplock groups took approximately seven hours 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 hours 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 ($H = 0.42$, d.f. = 1, $p = 0.52$) and aerobic incubation time ($H = 0.47$, d.f. = 1, $p = 0.49$).

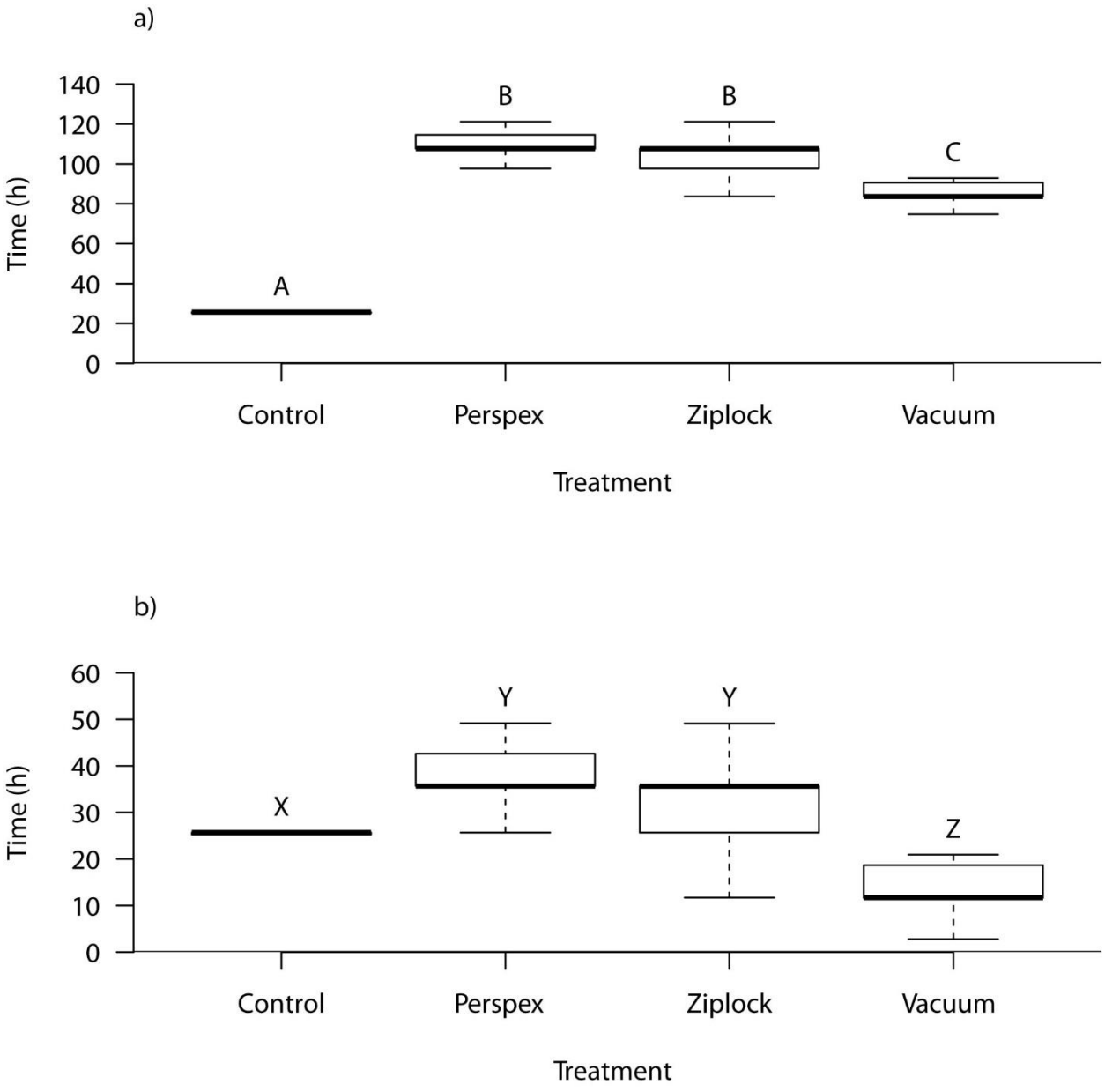


Figure 3.2. Latency till formation of the white spot on eggs measured as a) total time, or b) aerobic time. Eggs were subjected to either a control (normoxic) treatment (n = 76), or placed into Perspex chambers (n = 74) or ziplock bags (n = 70) into which nitrogen gas was pumped to exclude oxygen, or 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; $p \leq 0.05$).

3.4.2 Hatching

There was significant variation in hatching success among the treatment groups (X^2_{CMH} = 42.65, d.f. = 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 (Figure 3.3).

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; Figure 3.4). There was no significant difference between hatching success for the rotated and non-rotated eggs for the Perspex, ziplock, and vacuum treatments ($p = 0.14$, $p = 1$, $p = 0.35$; respective Fisher's exact tests; Figure 3.4).

There were between-treatment differences in the total time taken to hatch for the eggs incubated in the laboratory through to hatching ($F_{(3,6)} = 5.18$, $p < 0.05$; Figure 3.5). 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.

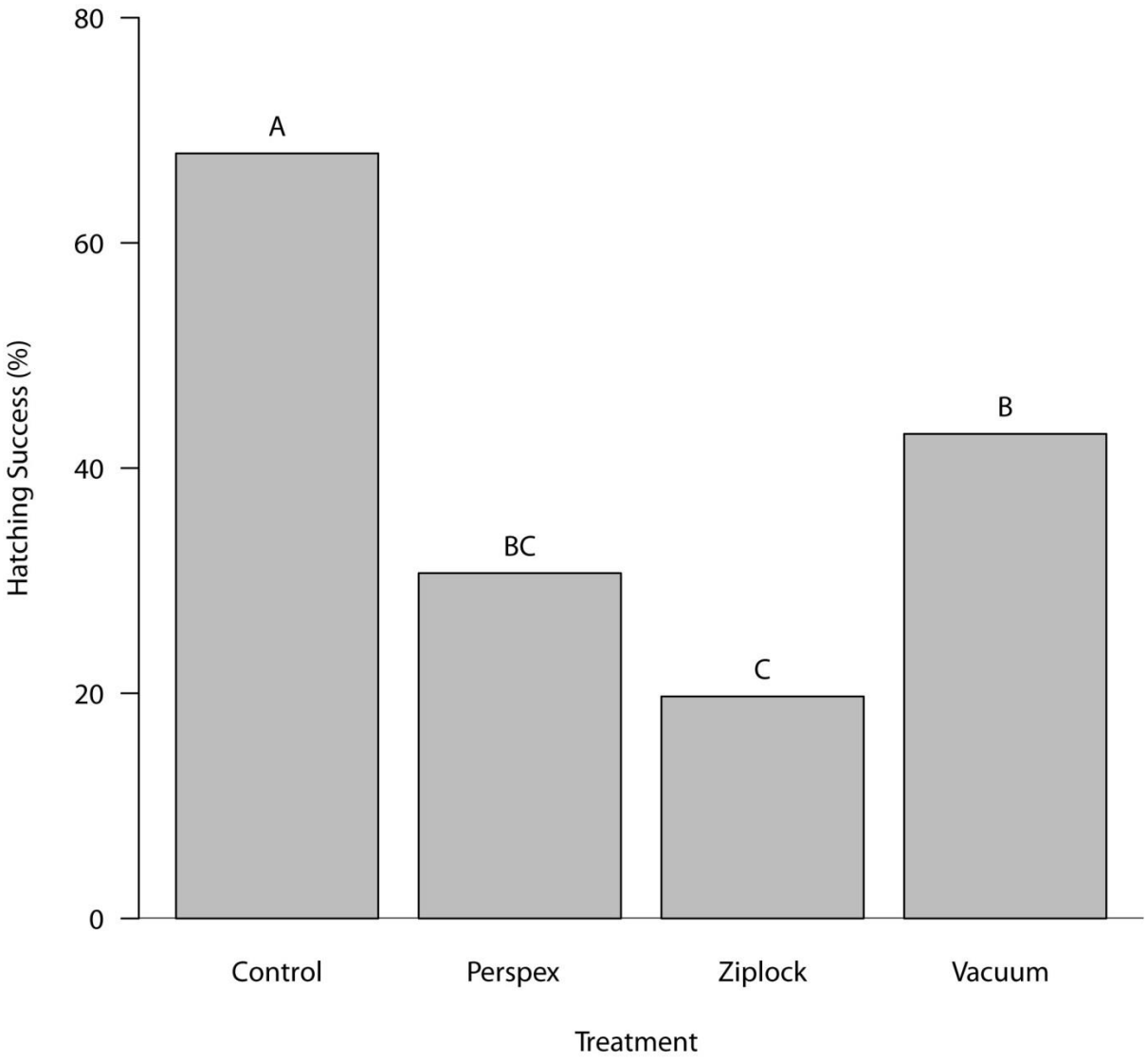


Figure 3.3. Total hatching success for olive ridley eggs placed into four treatments. Eggs were place into either a control treatment (N = 78), Perspex chambers (N = 75) or ziplock bags (N = 71) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags (N = 79). When letters 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; $p \leq 0.05$).

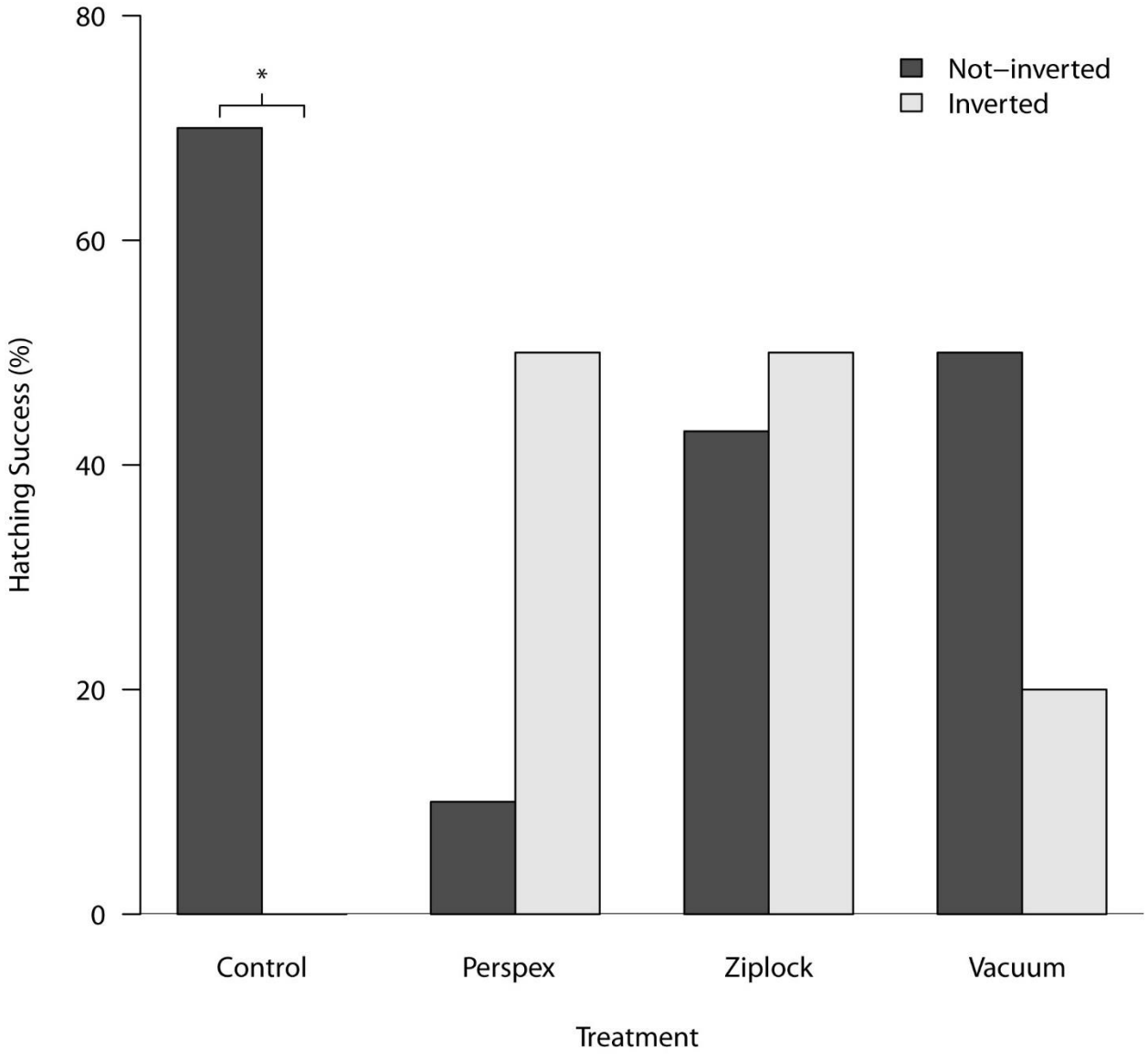


Figure 3.4. Hatching success for olive ridley eggs subjected to various treatments and incubated in the laboratory till hatching. Treatments were either control (normoxia; N = 20), Perspex chambers (N = 20) or ziplock bags (N = 15) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed 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 ($p \leq 0.05$).

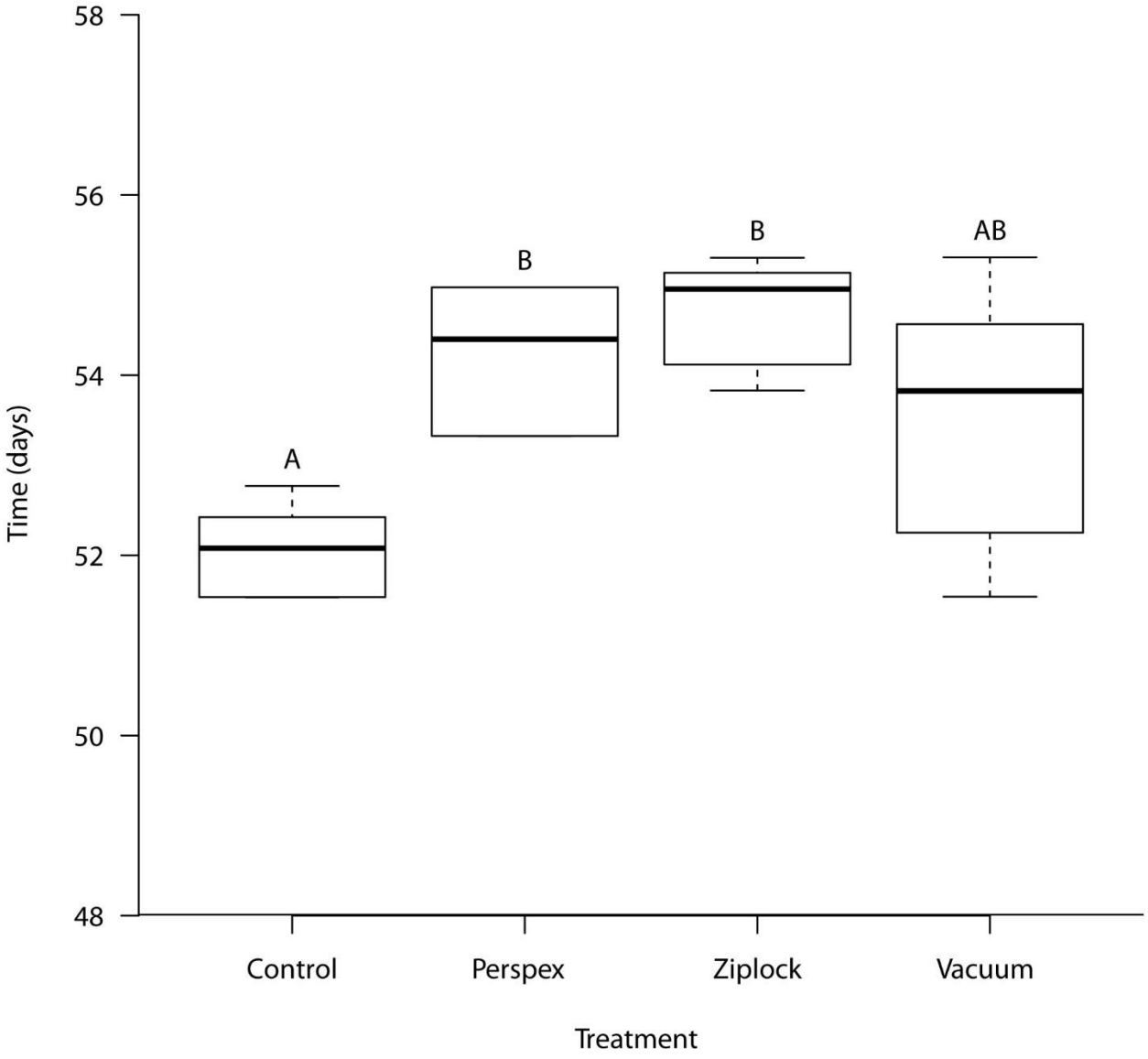


Figure 3.5. Incubation length of olive ridley eggs placed into four treatments immediately after oviposition. The four treatments lasted three days and consisted of; 1) a control treatment (n = 7), 2) Perspex chambers (n = 6) or 3) ziplock bags (n = 7) into which nitrogen gas was pumped to exclude oxygen, 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; $p \leq 0.05$).

3.4.3 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*; $p = 0.71$).

3.4.4 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 3.1). 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, d.f. = 1, p = 0.20).

Table 3.1. 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 placed 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.

3.5 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; Fordham *et al.* 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 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; Fordham *et al.* 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 hours (J. Wyneken, pers. comm.).

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.

3.6 Acknowledgements

S.W. was financially supported throughout his PhD with an Australian Postgraduate Award. This project was supported by the Holsworth Wildlife Research Endowment – Equity Trustees Charitable Foundation. We are grateful for the assistance provided by J. Black, B. Butler, L. Cruz, L. Domico, T. Dornfeld, L. Hackney, C. Hertz, L. McKenna, F. Paladino, G. Shillinger, E. Solano and J. Spotila at the Leatherback Trust and the Goldring-Gund Marine Biology Station. We thank the numerous Earthwatch Institute volunteers for their assistance in the field and laboratory. We appreciate the support of R. Zúñiga-Muños and the Area de Conservación Tempisque for our research. We thank P. Santidrián-Tomillo for assistance with permit applications. Y. Arguello-Gómez and C. Orrego helped with coordinating logistics in the field at

Playa Ostional. We are very appreciative and thankful for the support and use of facilities offered by R. Piedra and the staff at Parque Nacional Marino Las Baulas. Finally thanks to C. Neilson for invaluable assistance, inspiration and support in the field and laboratory.

3.7 References

Blanck, CE, Sawyer, RH (1981) Hatchery practices in relation to early embryology of the loggerhead sea turtle, *Caretta caretta* (Linné). *Journal of Experimental Marine Biology and Ecology* **49**, 163-177.

Booth, DT (2000) Incubation of eggs of the Australian broad-shelled turtle, *Chelodina expansa* (*Testudinata: Chelidae*), at different temperatures: Effects on pattern of oxygen consumption and hatchling morphology. *Australian Journal of Zoology* **48**, 369-378.

Booth, DT (2002) The breaking of diapause in embryonic broad-shell river turtles (*Chelodina expansa*). *Journal of Herpetology* **36**, 304-307.

Booth, DT, Feeney, R, Shibata, Y (2013) Nest and maternal origin can influence morphology and locomotor performance of hatchling green turtles (*Chelonia mydas*) incubated in field nests. *Marine Biology* **160**, 127-137.

Caillouet, CW, Shaver, DJ, Landry, AM (2015) Kemp's ridley sea turtle (*Lepidochelys kempii*) head-start and reintroduction to Padre Island National Seashore, Texas. *Herpetological Conservation and Biology* **10(Symp)**, 309-377.

Chan, E-H (1989) White spot development, incubation and hatching success of leatherback turtle (*Dermochelys coriacea*) eggs from Rantau Abang, Malaysia. *Copeia* **1989**, 42-47.

Chan, E-H, Salleh, HU, Liew, H-C (1985) Effects of handling on hatchability of eggs of the leatherback turtle, *Dermochelys coriacea*. *Pertanika* **8**, 265-271.

Crastz, F (1982) Embryological stages of the marine turtle *Lepidochelys olivacea* (eschscholtz). *Revista De Biologia Tropical* **30**, 113-120.

Deeming, CD (1991) Reasons for the dichotomy in egg turning in birds and reptiles. In 'Egg incubation: Its effects on embryonic development in birds and reptiles.' (Eds CD Deeming, MW Ferguson.) pp. 307-324. (Cambridge University Press: Cambridge, UK)

Dirzo, R, Young, HS, Galetti, M, Ceballos, G, Isaac, NJB, Collen, B (2014) Defaunation in the Anthropocene. *Science* **345**, 401-406.

Dodd, CK, Seigel, RA (1991) Relocation, repatriation, and translocation of reptiles and amphibians: Are they conservation strategies that work? *Herpetologica* **47**, 336-350.

Eckert, KL, Eckert, SA (1990) Embryo mortality and hatch success in *In situ* and translocated leatherback sea turtle *Dermochelys coriacea* eggs. *Biological Conservation* **53**, 37-46.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Ewert, MA (1991) Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In 'Egg incubation: ifs effects on embryonic development in birds and reptiles.' (Eds DC Deeming, MWJ Ferguson.) pp. 173-191. (Cambridge University Press: Cambridge, UK)

Ewert, MA, Wilson, DS (1996) Seasonal variation of embryonic diapause in the striped mud turtle (*Kinosternon baurii*) and general considerations for conservation planning. *Chelonian Conservation and Biology* **2**, 43-54.

Ferguson, MWJ (1985) Reproductive biology and embryology of the crocodilians. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 329-491. (John Wiley & Sons: New York)

Fordham, D, Georges, A, Corey, B (2006) Compensation for inundation-induced embryonic diapause in a freshwater turtle: Achieving predictability in the face of environmental stochasticity. *Functional Ecology* **20**, 670-677.

Fordham, DA, Georges, A, Corey, B (2007) Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise. *Aquaculture* **270**, 105-114.

Fuentes, MMPB, Fish, MR, Maynard, JA (2012) Management strategies to mitigate the impacts of climate change on sea turtle's terrestrial reproductive phase. *Mitigation and Adaptation Strategies for Global Change* **17**, 51-63.

Garcia, A, Ceballos, G, Adaya, R (2003) Intensive beach management as an improved sea turtle conservation strategy in Mexico. *Biological Conservation* **111**, 253-261.

Germano, JM, Bishop, PJ (2009) Suitability of amphibians and reptiles for translocation. *Conservation Biology* **23**, 7-15.

Hamann, M, Godfrey, M, Seminoff, J, Arthur, K, Barata, P, Bjorndal, K, Bolten, A, Broderick, A, Campbell, L, Carreras, C (2010) Global research priorities for sea turtles: informing management and conservation in the 21st century. *Endangered Species Research* **11**, 245-269.

Harry, J, Limpus, C (1989) Low-temperature protection of marine turtle eggs during long-distance relocation. *Wildlife Research* **16**, 317-320.

Hoegh-Guldberg, O, Hughes, L, McIntyre, S, Lindenmayer, DB, Parmesan, C, Possingham, HP, Thomas, CD (2008) Assisted colonization and rapid climate change. *Science* **321**, 345-346.

Kennett, R, Georges, A, Palmerallen, M (1993) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Kornaraki, E, Matossian, DA, Mazaris, AD, Matsinos, YG, Margaritoulis, D (2006) Effectiveness of different conservation measures for loggerhead sea turtle (*Caretta caretta*) nests at Zakynthos Island, Greece. *Biological Conservation* **130**, 324-330.

Leslie, AJ, Penick, DN, Spotila, JR, Paladino, FV (1996) Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990-1991. *Chelonian Conservation & Biology* **2**, 159-168.

Limpus, CJ, Baker, V, Miller, JD (1979) Movement induced mortality of loggerhead eggs. *Herpetologica* **35**, 335-338.

McDonald-Madden, E, Runge, MC, Possingham, HP, Martin, TG (2011) Optimal timing for managed relocation of species faced with climate change. *Nature Climate Change* **1**, 261-265.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Miller, JD (1999) Determining clutch size and hatching success. In 'Research and management techniques for the conservation of sea turtles.' (Eds KA Bjorndal, FA Abreu-Grobois, M Donnelly.) (IUCN/SSC Marine Turtle Specialist Group: Washington, DC)

Miller, JD, Limpus, CJ (1983) A method for reducing movement-induced mortality in turtle eggs. *Marine Turtle Newsletter* 10-11.

Mitchell, NJ, Rodriguez, N, Kuchling, G, Arnall, SG, Kearney, MR (2016) Reptile embryos and climate change: Modelling limits of viability to inform translocation decisions. *Biological Conservation* **204, Part A**, 134-147.

Parmenter, CJ (1980) Incubation of the eggs of the green sea turtle, *Chelonia mydas*, in Torres Strait, Australia: The effect of movement on hatchability. *Australian Wildlife Research* **7**, 487-491.

Pfaller, JB, Limpus, CJ, Bjorndal, KA (2009) Nest-site selection in individual loggerhead turtles and consequences for doomed-egg relocation. *Conservation Biology* **23**, 72-80.

Pike, DA, Roznik, EA, Bell, I (2015) Nest inundation from sea-level rise threatens sea turtle population viability. *Royal Society Open Science* **2**, 150127.

Pintus, KJ, Godley, BJ, McGowan, A, Broderick, AC (2009) Impact of clutch relocation on green turtle offspring. *Journal of Wildlife Management* **73**, 1151-1157.

R Core Team (2013) R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna, Austria

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Reina, RD (2014) The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. *Journal of Experimental Marine Biology and Ecology* **450**, 91-97.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**, e21038.

Rees, AF, Alfaro-Shigueto, J, Barata, PCR, Bjorndal, KA, Bolten, AB, Bourjea, J, Broderick, AC, Campbell, LM, Cardona, L, Carreras, C, Casale, P, Ceriani, SA, Dutton, PH, Eguchi, T, Formia, A, Fuentes, M, Fuller, WJ, Girondot, M, Godfrey, MH, Hamann, M, Hart, KM, Hays, GC, Hochscheid, S, Kaska, Y, Jensen, MP, Mangel, JC, Mortimer, JA, Naro-Maciel, E, Ng, CKY, Nichols, WJ, Phillott, AD, Reina, RD, Revuelta, O, Schofield, G, Seminoff, JA, Shanker, K, Tomás, J, de Merwe, J, Van Houtan, KS, Vander Zanden, HB, Wallace, BP, Wedemeyer-Strombel, KR, Work, TM, Godley, BJ (2016) Are we working towards global research priorities for management and conservation of sea turtles? *Endangered Species Research* **31**, 337-382.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Safina, C (2011) The 2010 Gulf of Mexico oil well blowout: A little hindsight. *PLOS Biology* **9**, e1001049.

Seddon, PJ, Griffiths, CJ, Soorae, PS, Armstrong, DP (2014) Reversing defaunation: Restoring species in a changing world. *Science* **345**, 406-412.

Shaver, DJ, Lamont, MM, Maxwell, S, Walker, JS, Dillingham, T (2016) Head-started Kemp's ridley turtle (*Lepidochelys kempii*) nest recorded in Florida: Possible implications. *Chelonian Conservation and Biology* **15**, 138-143.

Sieg, AE, Binckley, CA, Wallace, BP, Tomillo, PS, Reina, RD, Paladino, FV, Spotila, JR (2011) Sex ratios of leatherback turtles: Hatchery translocation decreases metabolic heating and female bias. *Endangered Species Research* **15**, 195-204.

Thompson, MB (1985) Functional significance of the opaque white patch in eggs of *Emydura macquarii*. In 'Biology of Australasian frogs and reptiles.' (Eds G Grigg, R Shine, H Ehmann.) pp. 387-395. (Surrey Beatty in association with The Royal Zoological Society of New South Wales: Chipping Norton, NSW)

Tuttle, J, Rostal, D (2010) Effects of nest relocation on nest temperature and embryonic development of loggerhead sea turtles (*Caretta caretta*). *Chelonian Conservation and Biology* **9**, 1-7.

Valverde, RA, Wingard, S, Gómez, F, Tordior, MT, Orrego, CM (2010) Field leathal incubation temperature of olive ridley sea turtle *Lepidochelys olivacea* embryos at a mass nesting rookery. *Endangered Species Research* **12**, 77-86.

Williamson, SA, Evans, RG, Reina, RD (2017) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Wyneken, J, Burke, TJ, Salmon, M, Pedersen, DK (1988) Egg failure in natural and relocated sea turtle nests. *Journal of Herpetology* **22**, 88-96.

Chapter 4. Embryonic development in the leatherback turtle and the effects of pre-ovipositional embryonic arrest on reproductive success.

Sean A. Williamson[1], Roger G. Evans[2] and Richard D. Reina[1]

[1] School of Biological Sciences, Monash University, Clayton, Victoria, Australia

[2] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton, Victoria, Australia

This manuscript is currently being prepared for submission to a peer-reviewed journal.

4.1 Abstract

Leatherback sea turtle egg clutches consistently experience lower hatching success (~50%) than those of other sea turtle species. The majority of embryonic death (>50%) occurs at early stages of development, possibly because embryos fail to break pre-ovipositional embryonic arrest after they are oviposited. Pre-ovipositional arrest is maintained by hypoxia in the oviduct and increased oxygen availability is the trigger that breaks the arrest after oviposition in all turtle species studied to date. Here, we examined the impact of pre-ovipositional embryonic arrest on reproductive success in leatherbacks. We conducted an 'ex-situ' incubator experiment and an 'in-situ' hatchery experiment. After oviposition eggs (N = 1005) were exposed to either normoxia (21% O_2), hyperoxia (32-42% O_2) for five days, or hypoxia (1% O_2) for three or five days. As has been found for other turtles, hypoxic incubation maintained embryos in arrest, equivalent to the time spent in hypoxia. However, extending arrest for five days resulted in greater early-stage death and a significant decrease in hatching success (68% lower than normoxic treatment). Eggs placed in incubators experienced greater hatching success than those placed into hatchery nests (67% vs 47%). We found no impact of hyperoxia on stage of embryonic death, hatching success, hatchling phenotype or fitness. Our findings indicate that delayed nesting and the subsequent extension of embryonic arrest may negatively impact embryonic development and therefore the reproductive success of leatherback turtles. They also indicate that incubation under hyperoxic conditions is unlikely to be a useful method to improve hatching success in this species.

Keywords: *Embryonic Development, Turtle Conservation, Pre-ovipositional Embryonic Arrest, Hypoxia, Hyperoxia, Delayed Nesting*

4.2 Introduction

Leatherback turtles (*Dermochelys coriacea*) are currently considered vulnerable to extinction at a global level (Wallace *et al.* 2013). There are many anthropogenic pressures currently impacting the species, including climate change, fisheries bycatch, human use, coastal development, pollution and pathogens (Wallace *et al.* 2011; Wallace *et al.* 2013). Particular populations are experiencing high levels of these pressures and this may be leading to extirpation (Chan and Liew 1996; Spotila *et al.* 2000; Santidrián Tomillo *et al.* 2008; Santidrián Tomillo *et al.* 2012; Tapilatu *et al.* 2013; Wallace *et al.* 2013). Much effort is focused upon conservation of populations at their nesting beaches, with particular emphasis placed on nest protection to increase hatchling production (Dutton *et al.* 2005; Santidrián Tomillo *et al.* 2007). However, leatherback turtles generally oviposit clutches of eggs of which only half survive to hatching (Chan *et al.* 1985; Whitmore and Dutton 1985; Leslie *et al.* 1996; Bilinski *et al.* 2001; Bell *et al.* 2003; Rafferty *et al.* 2011; Eckert *et al.* 2012; Rivas *et al.* 2016). Although variable, on average this is considerably less than all other sea turtles, which generally have a hatching success of 70% or more (Whitmore and Dutton 1985; Miller 1997; Hewavisenthi and Parmenter 2002; Pike 2008; Dornfeld *et al.* 2015). Understanding why leatherback turtles commonly experience low hatching success could offer potential solutions to managers and conservationists to improve hatchling production at nesting sites. However, the reasons for the disparity in hatching success between leatherbacks and other species of sea turtle are still unclear.

There are two possible causes of low hatching success for an oviparous species; either low rates of egg fertilisation, or high rates of embryonic mortality (Bell *et al.* 2003). Egg fertilisation does not appear to be a contributing factor for leatherbacks (Whitmore and Dutton 1985; Bell *et al.* 2003) and there is little empirical evidence that it has a major effect on hatching success in other

sea turtle species. However, there are high levels of embryonic mortality, and usually at least half of the embryos that fail to hatch die at an early stage of development (Whitmore and Dutton 1985; Eckert and Eckert 1990; Bell *et al.* 2003; Rafferty *et al.* 2011). Recent evidence indicates that longer inter-nesting periods result in an increase in the proportion of embryos that die at this early embryonic stage (Rafferty *et al.* 2011). During a nesting season leatherback females average six nests; one every nine to ten days (inter-nesting interval), and they migrate to nesting beaches every two to four years (Boulon *et al.* 1988; Tucker and Frazer 1991; Hughes 1996; Reina *et al.* 2002; Rivalan *et al.* 2005; Eckert *et al.* 2012; Rivas *et al.* 2016), with their remigration interval influenced by climate and foraging opportunities (Saba *et al.* 2007; Reina *et al.* 2009). There is evidence from other sea turtle species that once the first clutch is oviposited the next clutch of eggs is then ovulated, usually within two days (Licht 1980, 1982; Licht *et al.* 1982; Owens and Morris 1985; Rostal *et al.* 1996; Valverde 1996; Rostal *et al.* 2001). Fertilisation of the ova occurs after ovulation and is usually complete within the first 72 hours after the last nest was oviposited (Miller 1997; Eckert *et al.* 2012; Abella *et al.* 2017). The albumen is then secreted around the fertilised embryo and yolk, and the clutch moves down the oviduct (Miller 1985). Finally, the eggshell is secreted and complete within one week of ovulation (Miller 1985). Therefore, eggs are fully calcified and sitting in the oviducts for two to three days before they are laid nine or ten days after the previous clutch.

Turtle embryos arrest development as a gastrula after ovulation and whilst still in the hypoxic oviduct (Ewert 1985; Miller 1985; Rafferty and Reina 2012; Rafferty *et al.* 2013). When nesting is delayed, and the interval between clutches is extended, eggs spend longer in this pre-ovipositional embryonic arrest (Risley 1944; Lynn and von Brand 1945). The arrest is maintained by hypoxia (Kennett *et al.* 1993; Rafferty *et al.* 2013; Rings *et al.* 2015; Williamson

et al. 2017), and when arrest is extended by hypoxic incubation after oviposition, early-stage embryonic death can increase (Rings *et al.* 2015). However, it remains to be tested whether hypoxia maintains embryonic arrest in leatherback turtles and whether increased duration of arrest results in a decreased hatching success or even an increase in the proportion of early stage death.

If embryonic arrest is a contributing factor to early-stage death in leatherback turtles (Rafferty *et al.* 2011), it could be a consequence of embryos failing to break arrest and recommence development following oviposition. Increased oxygen availability when eggs move from the hypoxic oviduct to the normoxic nest is the trigger that breaks the arrest in the 12 hours or so after oviposition (Kennett *et al.* 1993; Fordham *et al.* 2007; Rafferty *et al.* 2013; Rings *et al.* 2015; Williamson *et al.* 2017). Therefore, given that oxygen stimulates development, we hypothesise that hyperoxia (high levels of oxygen availability) may result in more embryos breaking from embryonic arrest and developing past early-stages of development after oviposition, thereby reducing early-stage embryonic death. We have already shown that hyperoxic incubation has no detectable negative impacts upon two other reptiles; flatback turtles (Rings *et al.* 2015), and saltwater crocodiles (Williamson et al. unpublished data). If hyperoxic incubation can mitigate early-stage embryonic death in leatherback turtles it could potentially increase hatching success. Hyperoxic incubation could therefore offer a potential option for managers and conservationists to use in leatherback turtle hatcheries to increase hatchling production.

Considering the potential importance of pre-ovipositional embryonic arrest for the reproductive ecology of leatherback turtles we aimed to determine whether 1) hypoxic incubation after oviposition can be used to extend arrest in this species, 2) increased duration of arrest results in

increased early-stage embryonic death and/or decreased hatching success, 3) hyperoxic incubation following oviposition decreases the proportion of early-stage death and increases hatching success, and 4) hyperoxia can be administered successfully to incubating nests 'in-situ' for potential wide-scale management use. To do this, we employed an ex-situ incubation experiment to assess the effect of hypoxia and hyperoxia for varying lengths, and an in-situ experiment to assess the impact of hyperoxic incubation for nests in a hatchery.

4.3 Materials and methods

4.3.1 Regulatory approval

Monash University's School of Biological Sciences Animal Ethics Committee approved all experimental procedures (Approval BSCI/2016/13). The research was conducted under a scientific permit issued by the Costa Rican Ministerio Del Ambiente y Energia, Sistema Nacional de Áreas de Conservación, Área de Conservación La Amistad Caribe (RESOLUCIÓN SINAC-ACLAC-PIME-VS-R-022-2016).

4.3.2 Collection of eggs for the ex-situ experiment

Eggs were collected from five nesting leatherback turtles (n = 20 eggs per female) using gloved hands. The eggs were immediately vacuum sealed in plastic bags (Airlock, Australia) using a hand pump vacuum (Airlock, Australia) and transported to the laboratory (100 m to 1 km distant; depending on nesting location).

4.3.3 Ex-situ experimental design

Once the eggs arrived at the laboratory they were randomly divided amongst one of four oxygen treatments (*n* = 25 eggs per treatment); a 5-day normoxia, 5-day hyperoxia, 3-day hypoxia or 5-day hypoxia experiment. All eggs were placed into airtight Perspex (clear polymethyl methacrylate plastics, also called Plexiglass or Lucite) chambers (Resi-Plex Plastics, North Geelong, Australia). There were three chambers used per treatment to account for any potential variation between vessels. The eggs rested on a mesh wire which was raised above 10 mL of distilled water within each chamber. Pure nitrogen gas was used for the hypoxic treatments and 42% O_2 in nitrogen was used for the hyperoxic treatment (INFRA G.I., San Jose, Costa Rica). Gas was humidified by bubbling it through water prior to it flowing through the chamber using inflow and outflow gas valves. Each gas was administered for three minutes at a flow rate of 8 L/min through the respective hypoxic or hyperoxic chamber. The normoxic treatment had ambient air circulated through the chamber for three minutes. Oxygen sensors (Analytical Industries, Pomona, CA) and a data collection device (Pasco, Roseville, CA) were used to monitor the PO_2 of gas leaving the outflow valve for each chamber. The chambers were then sealed and placed into incubators (GQF HovaBator model 1632; Grandview Management, Baldivis, Australia) set to 30 ± 1 ºC. Every 24 hours the gas treatment was reapplied to the chambers as described above.

4.3.4 Ex-situ egg incubation

After three (three-day hypoxia treatment) or five days (remaining three treatments), eggs were carefully removed from their Perspex chambers and placed in sand (7 ± 2% gravimetric water content) within normoxic incubators set to 30 ± 1 ºC. The eggs were monitored three times daily

for formation of the opaque white spot on the upper surface. The white spot indicates where the shell and embryonic membranes have fused and is an indication of development recommencing after oviposition (Abella *et al.* 2017). While the eggs were being removed from their chambers, two eggs from the hyperoxia treatment were inadvertently rotated. Those two eggs had already formed white spots but were subsequently excluded from hatching success calculations.

4.3.5 Ex-situ hatching and embryonic death

The time and date of hatching (emergence of the neonate from the egg) were recorded. Eggs suspected of containing dead embryos were monitored over a few days by carefully candling the eggs (using a head torch shone through the egg) to assess embryonic development. Eggs that failed to hatch or were determined to have died (no signs of embryonic development, fungal growth or abnormal coloration) were removed from the incubators. Dead eggs were opened and embryonic stage of death was assessed using Miller's (1985) 31-stage and Leslie et al.'s (1996) 4-stage developmental chronologies. Hatching success (%) for each treatment was calculated as the number of eggs to hatch of the total number of eggs for that treatment. Hatchlings were kept in a separate incubator for two days to allow absorption of yolk before their morphology and fitness was assessed.

4.3.6 Ex-situ hatchling morphology and fitness

Hatchling mass (g) was recorded using a spring scale (± 0.1 g). Head width, and carapace width and length (mm) were measured using a digital dial caliper (± 0.01 mm). Hatchlings were allowed to acclimate to 30 ± 1 ºC in the incubator for at least five minutes before crawling speed and self-righting ability were tested. The crawl speed was assessed using a 1 m gutter dug into sand near the high tide line on the beach. The gutter sloped downwards toward the tideline (~5º

angle below the horizontal). Sand in the bottom of the gutter was smoothed by hand prior to each test. A hatchling was then placed at the upper end of the gutter and the time taken to start crawling and the time taken to reach the end of the gutter were recorded. Crawling speed for each hatchling was tested three times. The self-righting ability of each hatchling was tested using a methodology similar to that employed by Booth *et al.* (2013). Each hatching was flipped onto its carapace on flat sand and the time taken for it to start moving, and total time for the hatchling to flip back onto its plastron, was recorded. A trial was abandoned and the hatchling placed back onto its plastron if it had not successfully righted itself after one minute. The righting trial was repeated until three successful righting attempts had been made or a total of six trials had been conducted for that hatchling. This enabled us to then calculate a 'righting propensity' score for each hatchling (Table 4.1).

Table 4.1. Self-righting propensity score for ex-situ incubated hatchlings.

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

Based on Booth *et al*. 2013

4.3.7 Collection of eggs for the in-situ experiment

Clutches of leatherback turtle (*Dermochelys coriacea*) eggs (Clutches N = 14; eggs N = 905) were collected at the time of laying between the 9th of June and 16th of June 2016 at Reserva

Pacuare on the Caribbean coast of Costa Rica. The clutches were collected into plastic bags using gloved hands and the time of oviposition for the first and last egg from each clutch was recorded. The eggs were then transported a short distance (50 m to 5 km depending upon nesting location) to a hatchery close to the station at Reserva Pacuare.

4.3.8 In-situ experimental design

Once each clutch had arrived at the hatchery the eggs were split randomly between two nest cavities. Nests cavities were constructed approximately 1.2 m apart from one another within a hatchery at the back of the beach berm. A treatment was randomly assigned to each nest; either normoxia (nests $N = 10$, eggs $N = 453$) or hyperoxia (nests $N = 10$, eggs $N = 452$). Nest cavities were excavated by hand to 70 cm total depth, taking care to mimic as closely as possible the natural shape of nests of *D. coriacea*.

Prior to placement of eggs into the cavity, a hollow plastic practice golf ball with many holes in the plastic was placed at the bottom of the nest cavity. A plastic gas tube was inserted through one of the holes and into the centre of the ball. The gas tube ran from the bottom of the nest, up the side of the nest chamber and to the back of the hatchery, where it was attached to either a normoxic (21% O_2) or hyperoxic (42% O_2) gas line. The eggs were then gently placed on top of the golf ball. The shelled albumen gobs (SAGs) from each clutch were also evenly divided between each nest and placed on top of the eggs.

Oxygen sensors (Analytical Industries, Ponoma, CA) were placed at the top of a subsample of nests ($N = 7$) from both the normoxic ($n = 3$) and hyperoxic treatments ($n = 4$). The wire for the oxygen sensor was led out of the nest chamber next to the gas line. The connector part at the end

of the wire was placed into a small plastic container for protection from moisture and sun exposure. All nests were then filled with sand to mimic natural nests laid on the beach.

Each night we aimed to collect at least two clutches. This enabled us to place half the eggs from one clutch with half the eggs from another clutch into each nest cavity. Eggs from the two clutches were evenly spread throughout the nest. However, on some nights we were unable to obtain multiples of two clutches during our patrols of the beach. On these occasions we split individual clutches (Clutches $N = 6$; eggs $N = 343$) into two nest cavities without placing eggs from another clutch into the same nests.

A gas cylinder with 42% O_2 in nitrogen (INFRA G.I., San Jose, Costa Rica) was placed at the back of the hatchery. This provided the gas mixture for the hyperoxic treatment that was applied to half of the nests. The gas cylinder was connected to a cylinder regulator and needle flow control valve. A plastic gas tube connected the flow valve to a low voltage timed solenoid valve powered by a 12 V battery and set to open for 3 minutes every 30 minutes. The solenoid valve was housed within a waterproof plastic container and connected to a plastic gas tube which ran out of the container to a water chamber to humidify the gas as it passed through. Another gas line then ran from the water chamber and connected to a gas line splitter which separated into ten gas lines, each running to a separate nest in the hyperoxic treatment group. Each gas line was equal in length (10 m) to ensure that there was no pressure differential between different lines. Approximately two meters prior to the line reaching the golf ball in the nest a plastic 2-way stopcock valve was placed to enable specific nests to have their gas flow turned off or on as required. The needle flow control valve was used to ensure the volume flowing out the end of each gas line was always kept at four litres per minute when the lines were open.

A 12 V battery-powered air pump was used to provide the gas mixture for the normoxia treatment. The pump was turned on for three minutes every half an hour using an electronic timer switch and circulated atmospheric air (~21% O_2) into a gas line that was humidified by pumping it through a water chamber. As for the hyperoxic treatment, a gas line splitter was then used to run ten separate lines to the golf balls in the normoxic nests. However, instead of a 2-way stopcock valve being placed into the line, a plastic 2-way flow control valve was used. These flow control valves were then adjusted to ensure the flow rate out of each line was kept at four litres per minute when the lines were open.

The PO_2 of gas in a subset of both the normoxic (n = 3) and hyperoxic nests (n = 4) was monitored by connecting the oxygen sensor (Analytical Industries, Pomona, CA) in each nest to a data collection device (Pasco, Roseville, CA). Gas treatments were applied to each nest for five days after burial. The fractional volume of oxygen in the hyperoxic treatment nests was (mean ± standard error) 31.7 ± 0.8% O_2, whilst the normoxic nests remained at 20.9 ± 0.4% O_2.

4.3.9 Nest excavation

Each nest was excavated two days after the first hatchling emerged or if no hatchlings had emerged after 70 days of incubation. Hatching success was calculated as the proportion of eggs that hatched of the total number of eggs in the nest. The unhatched eggs remaining in the nest were then opened and the stage of each embryo was identified according to Leslie *et al.*'s (1996) field-staging method. Rafferty *et al.* (2011) describe in detail how this method classifies Miller's (1985) 31-stage developmental chronology into four broader stages of development.

4.3.10 Statistical analysis

For the ex-situ eggs, to assess the influence of different post-oviposition oxygen conditions on the proportion of eggs to form white spots and the number of eggs to die at each of Leslie et al.'s (1996) four developmental stages a Fisher's exact test was used to determine between treatment variation. Fisher's exact test was also used to examine the between-treatment variation in the proportion of eggs to die at Leslie et al.'s (1996) Stage 0 of the total embryonic death. Data for latency until white spot formation (both total time and aerobic incubation time), hatchling morphology and fitness traits violated the assumption of normality (Shapiro-Wilk test). Therefore, between-group differences for these data were assessed using the Kruskal-Wallis test and Nemenyi post-hoc tests. Variation in hatching success among the four treatments was assessed using a Cochran-Mantel-Haenszel (CMH) test (adjusting for clutch identity) with Bonferroni corrections for pair-wise comparisons.

For the in-situ experiment, to determine whether there was a difference between the hyperoxic and normoxic nests in mean proportion of embryonic deaths at each developmental stage, mean hatching success and mean time taken to for the first hatchling to emerge from the nest since oviposition two-way analyses of variance (ANOVAs) were used. Treatment group and nest size (half vs full nests) were the two independent factors. Homoscedasticity and normality of the two dependent variables were assessed using the Filgner-Killen and Shapiro-Wilk tests. Tukey's HSD test was used for post-hoc analysis for the two-way ANOVAs. To determine whether the total proportion of eggs that died at each of Leslie et al.'s (1996) four developmental stages differed between the two treatments a chi-squared test was used.

Finally, to compare the total hatching success between all normoxia and hyperoxia treatments (with the in- and ex-situ data combined) and between all the in-situ and ex-situ eggs (with the normoxic and hyperoxic data combined for each) chi-squared tests were used. All values are presented as mean ± standard error or, when normality was violated, median (range). Two-tailed $p \leq 0.05$ was considered statistically significant. All statistical analyses were conducted using R software (R Core Team 2016).

4.4 Results

4.4.1 Ex-situ experiment

There was no major difference between eggs incubated hyperoxic and normoxic treatments for the ex-situ experiment. The timing of egg development (white spot formation and stage of embryos) and hatching was similar between these two treatments, as was the hatching success. However, there was a difference between these two treatments (hyperoxia and normoxia) and the two hypoxic treatments in both egg development and hatching. Finally, there was no difference between the hatchlings from the different treatments.

4.4.1.1 Egg development

There was no significant between-group variation in the proportion of ex-situ incubated eggs to form white spots (Fisher's exact test; $p = 0.06$), with all forming white spots except for three eggs in the five-day hypoxia treatment. However, there was significant between-treatment variation in the latency to white spot formation ($H = 81.43$, d.f. = 3, $p < 0.0001$; Figure 4.1a). White spot development was delayed by hypoxia relative to normoxic eggs, with the delay being approximately equivalent to the time spent in hypoxia. That is, eggs in the three-day hypoxic

group took approximately two and a half days longer to form white spots than the normoxic group, while eggs from the five-day hypoxic group took approximately five days longer to form white spots than the normoxic group. Latency to white spot formation was similar in eggs from the normoxic and hyperoxic treatments (Figure 4.1a). There was still a significant between-group difference in latency to white spot formation even when the time eggs spent in hypoxia was accounted for (i.e. when assessing aerobic latency; H = 9.98, d.f. = 3, $p = 0.02$; Figure 4.1b). The aerobic latency to white spot formation was calculated by subtracting the time spent in hypoxia from the total time elapsed between oviposition and the appearance of the white spot. Aerobic latency was approximately 13 hours shorter in the three-day hypoxia group than in the normoxic group.

4.4.1.2 Hatching and hatchling traits

Hatching success varied significantly among the treatment groups ($X^2_{CMH} = 28.78$, d.f. = 3, $p < 0.0001$; Figure 4.2) with no significant two-way interaction with clutch identity (Woolf test $X^2 = 2.17$, d.f. = 4, $p = 0.70$). The normoxic and hyperoxic treatments had greater hatching success (72% and 61% respectively) than the five-day hypoxic treatment (4%). Hatching success in the three-day hypoxic treatment (32%) was not significantly different to any of the other three treatments. Eggs from the three-day hypoxia treatment and the one egg that hatched from the five-day hypoxia treatment took approximately three days longer on average to hatch than the normoxic and hyperoxic treatments (Table 4.2). However, this variation in incubation duration was not statistically significant ($F_{(3,27)} = 3.76$, $p = 0.08$). There was no significant between-group variation in hatchling morphology or fitness traits (Table 4.2; $p > 0.05$).

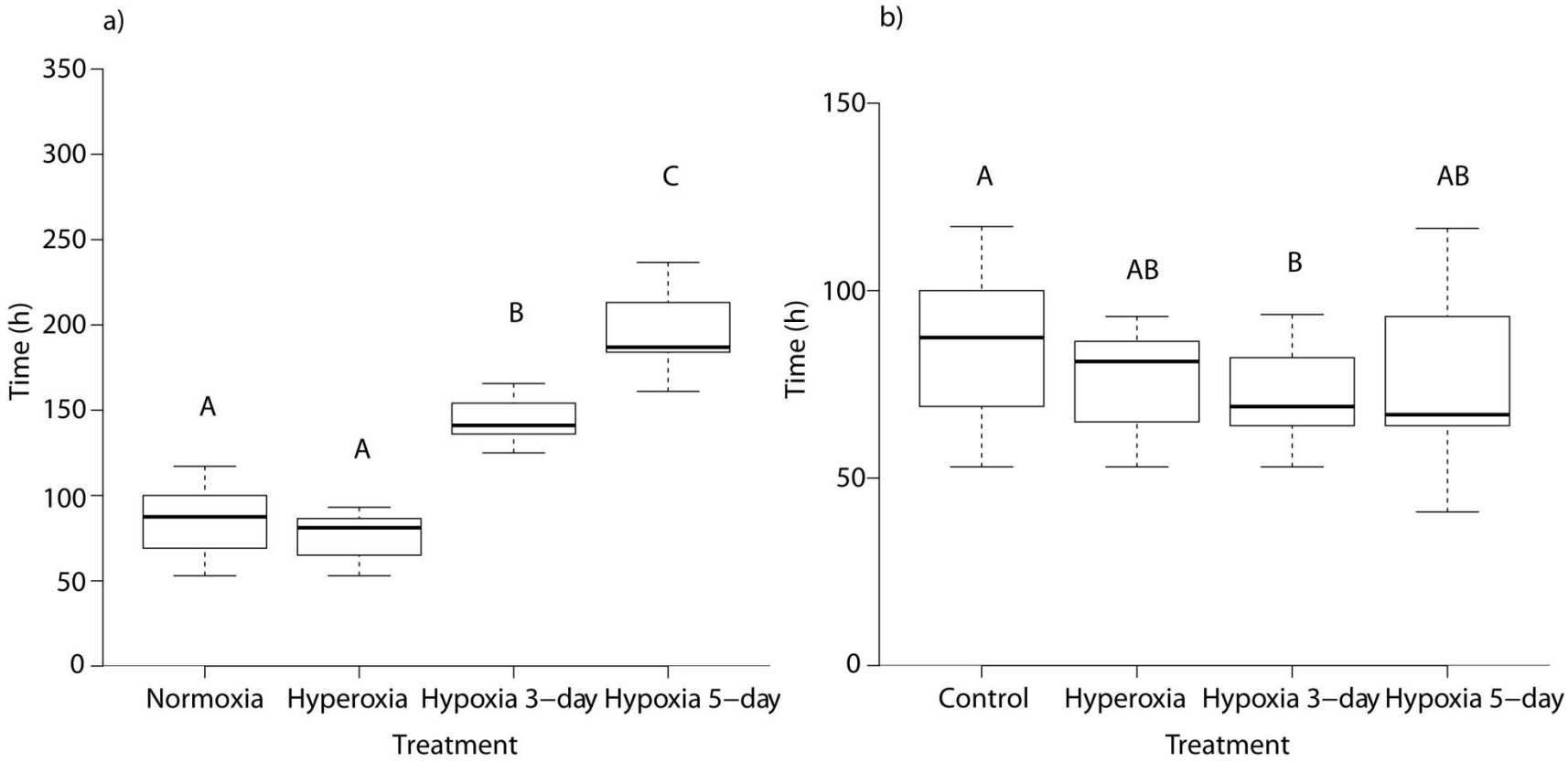


Figure 4.1. Latency from oviposition to formation of the white spot on eggs in a) total time and b) aerobic time. Leatherback turtle eggs (N = 100) were incubated in either normoxia (21% O_2) for five days, hyperoxia (42% O_2) for five days, or hypoxia (1% O_2) for three or five days (n = 25). Aerobic time is the total time from oviposition excluding time spent in hypoxia. Boxplot centre lines show medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. If 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; $p \leq 0.05$).

Table 4.2. Traits of leatherback turtle hatchlings from various ex-situ incubation treatments.

	Normoxia	Hyperoxia (5-days)	Hypoxia (3-days)	Hypoxia (5-days)	Test statistic	*P* value
No. of hatchlings	18	14	8	1	$X^2_{CMH} = 28.78$	<0.0001
Hatching time (d)	60.6 ± 0.2	59.9 ± 0.4	63.2 ± 1.0	63.2	$F_{(3, 27)} = 3.76$	0.08
Mass (g)	44.0 (35.3 – 48.4)	43.8 (31.1 – 48.0)	43.5 (33.0 – 48.1)	39.0	$H_{(3)} = 2.16$	0.54
Carapace Length (mm)	61.6 (54.6 - 64.52)	60.7 (54.6 – 63.7)	58.5 (51.5 – 63.5)	59.0	$H_{(3)} = 4.16$	0.24
Carapace Width (mm)	42.2 (39.1 – 44.5)	42.0 (38.7 – 44.7)	42.0 (36.9 – 43.6)	40.7	$H_{(3)} = 2.15$	0.54
Head Width (mm)	17.8 (16.5 – 18.4)	17.7 (16.6 – 18.3)	17.6 (15.8 – 18.4)	17.0	$H_{(3)} = 2.81$	0.42
Self-righting time (s)	3.2 (1.3 – 42.7)	3.0 (1.7 – 16.0)	4.3 (1.7 – 11.3)	4.3	$H_{(3)} = 0.29$	0.96
Righting propensity	6.0 (0 – 6)	4.5 (0 – 6)	5.5 (0 – 6)	6	$H_{(3)} = 4.42$	0.22
Crawl speed (cm/s)	4.0 (1.0 – 6.4)	3.3 (0.9 – 5.5)	3.3 (1.9 – 6.4)	1.6	$H_{(3)} = 3.89$	0.27

Eggs (N = 98) were incubated in either normoxia (control; n = 25), hyperoxia (42% O_2; n = 23) for five days, or hypoxia (1% O_2) for three or six days (n = 25). Following their respective treatments all eggs were incubated in normoxia until hatching. Hatching time data are mean ± standard error, data for all other hatchling traits violated normality, so is presented as median (range).

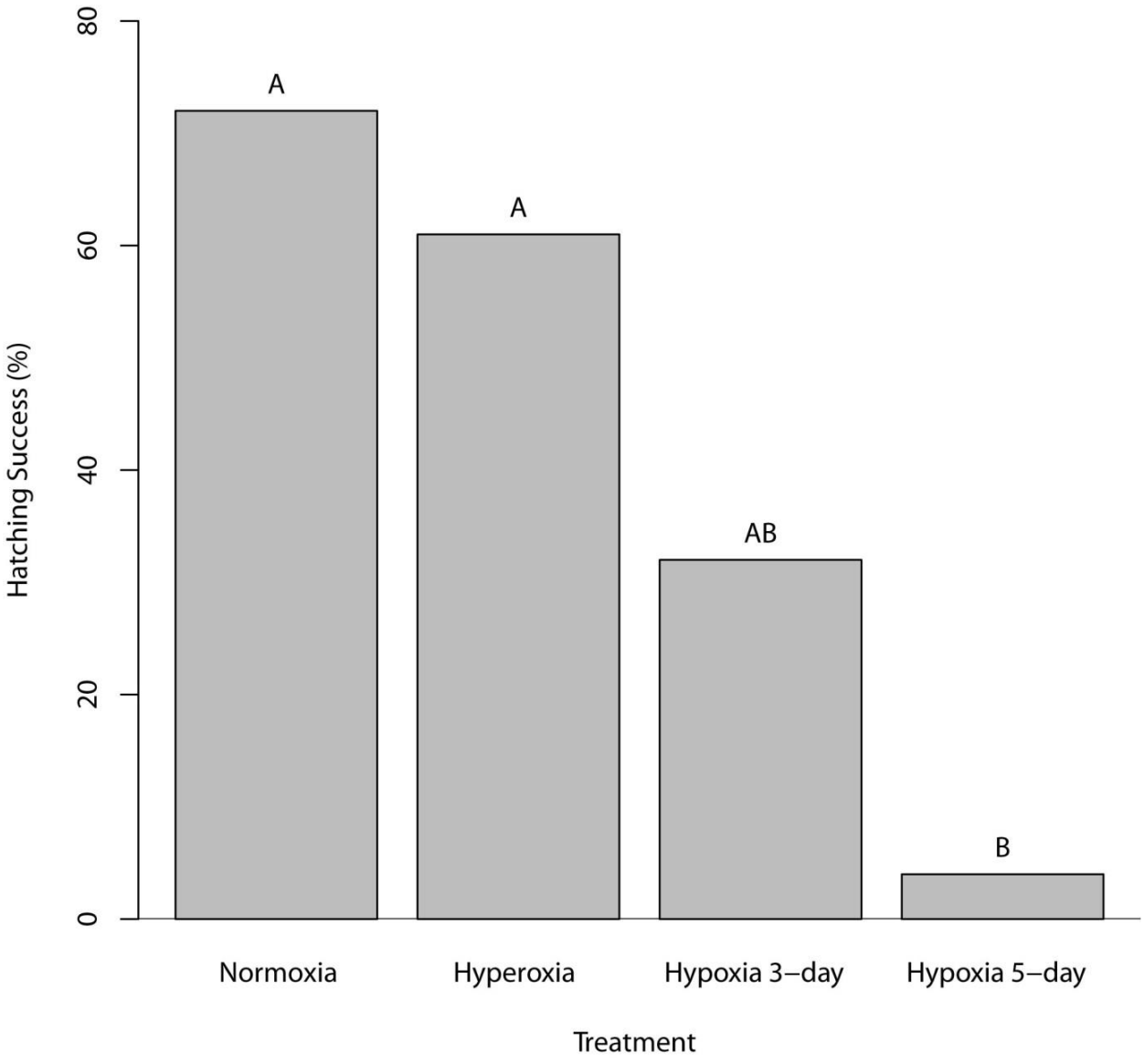


Figure 4.2. Proportion of leatherback turtle eggs to hatch (%) after placement in various ex-situ incubation treatments. Eggs (N = 98) were incubated in either normoxia (21% O_2; n = 25), hyperoxia (42% O_2; n = 23) for five days, or hypoxia (1% O_2) for three or five days (n = 25). Following their respective treatments all eggs were incubated in normoxia until hatching. When letters above each bar are the same, there was no significant between-group difference in hatching success (Bonferroni corrected chi-squared test with six pair-wise comparisons; $p \leq 0.05$).

4.4.1.3 Embryonic mortality

There was no significant between-group variation in the number of embryos that died at each of Leslie et al.'s (1996) four developmental stages (Fisher's exact test; $p = 0.08$). However, there was a tendency for between-group variation in the proportion of stage zero death (Fisher's exact test; $p = 0.08$). There was more stage zero death in the five-day hypoxia treatment than in the other treatments (Figure 4.3).

4.4.2 In-situ experiment

Increasing oxygen availability during the start of incubation in-situ appeared to have no impact on leatherback reproductive success. That is, there was no difference in hatching success, incubation time, and stage of embryonic death, between the normoxic and hyperoxic nests.

4.4.2.1 Hatching

Mean hatching success did not significantly differ between the normoxic and hyperoxic nests (41 ± 9% and 40 ± 9% respectively; $F_{(1,16)} = 0.01$, $p = 0.91$). Nests that were buried with only a single half-clutch had reduced hatching success when compared with nests buried with two half-clutches inside (30 ± 12% and 55 ± 11% respectively). However, this difference was not statistically significant ($F_{(1,16)} = 4.20$, $p = 0.06$). Furthermore, there was no significant two-way interaction between clutch size and treatment on hatching success ($F_{(1,16)} = 0.04$, $p = 0.85$). The average time until first emergence of hatchlings from the nest did not vary significantly between the normoxic and hyperoxic nests (61 ± 1 days and 60 ± 1 days respectively; $F_{(1,13)} = 1.88$, $p = 0.19$). However, hatchlings from half clutch nests took significantly longer until first emergence than hatchlings from full clutch nests (62 ± 1 days and 59 ± 1 days respectively; $F_{(1,13)} = 8.76$, p

= 0.01). The trend for an impact of nest size on hatching success did not vary between treatments, as demonstrated by the absence of a two-way interaction between treatment and nest size ($F_{(1,13)} = 0.43$, $p = 0.52$).

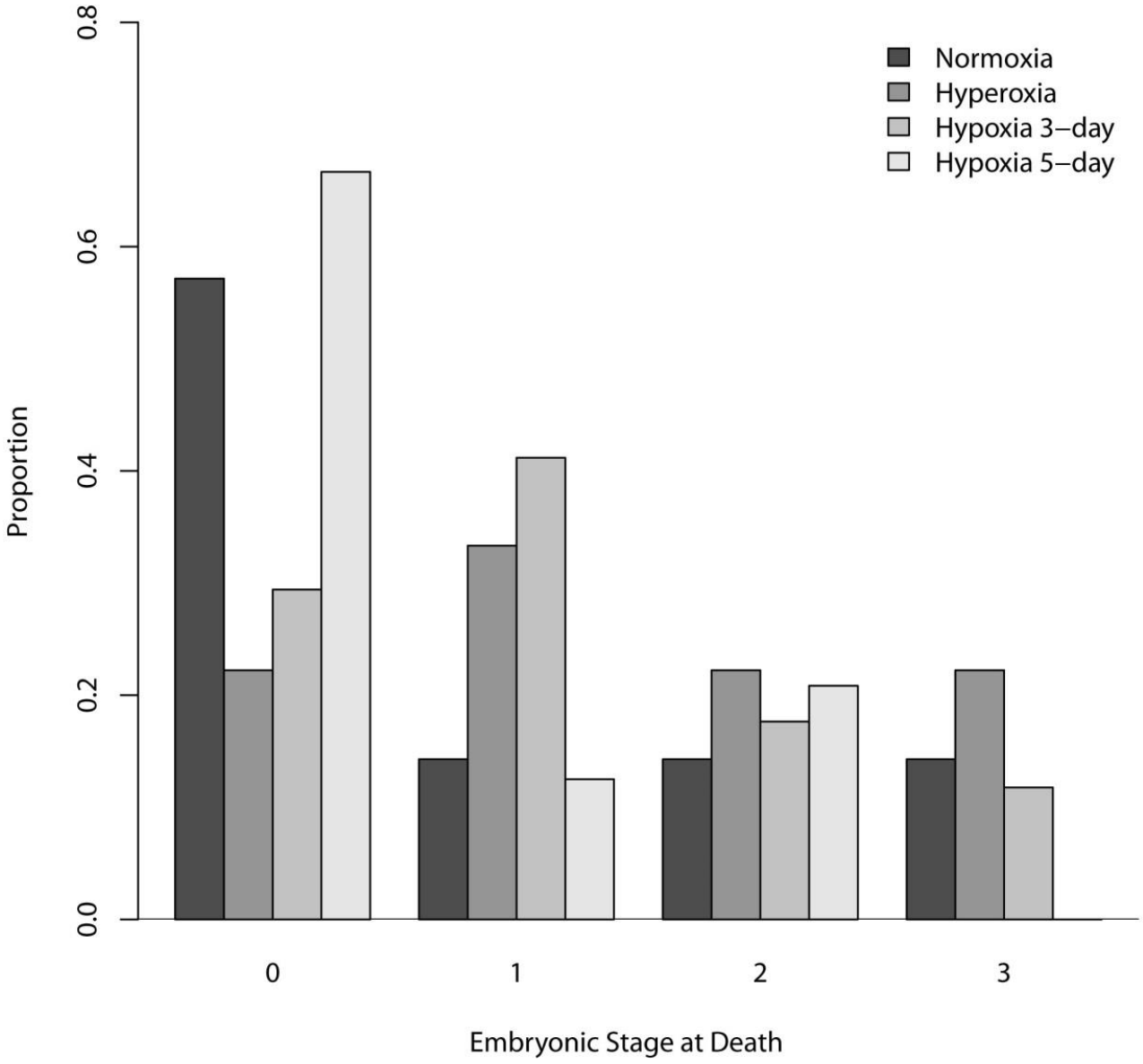


Figure 4.3. Proportion of embryonic death at four stages of development in leatherback turtle eggs. Eggs ($N = 98$) were incubated in either normoxia (21% O_2), hyperoxia (42% O_2) for five days, or hypoxia for three or five days ($n = 23$ - 25). Following their respective treatments all eggs were incubated in normoxia until hatching. Embryos were staged according to Leslie et al.'s (1996) 4-stage developmental chronology.

4.4.2.2 Embryonic death

The number of embryos that died at each developmental stage did not significantly differ between the two treatments (X^2 = 7.57, d.f. = 4, p = 0.11). However, there was a significant difference in the mean proportion of death at the various stages of development ($F_{(3,64)}$ = 44.69, p < 0.0001). A greater proportion of embryos died at stage zero of development than at the other three stages of development (Figure 4.4). There was no significant between-treatment difference in the proportion of embryos that died at each developmental stage ($F_{(3,64)}$ = 0.19, p = 0.89). There was also no significant difference in the proportion of deaths at each stage between half-clutch and full-clutch nests ($F_{(3,64)}$ = 0.58, p = 0.63).

4.4.3 Collective hatching success

When the ex-situ and in-situ data were combined there was no significant difference in the hatching success between hyperoxia and normoxic treatments (X^2 = 0.56, d.f. = 1, p = 0.45). In total, 49.0% (234 / 478) of the eggs exposed to normoxia hatched compared to 46.5% (221 / 475) of the eggs exposed to hyperoxia. However, there was a significant difference in the collective hatching success between eggs buried in the hatchery (in-situ experiment) and those placed in incubators (ex-situ experiment) when excluding the ex-situ hypoxic treatments (X^2 = 6.48, d.f. = 1, p = 0.01). In total, 47% (423/905) of in-situ eggs hatched compared to 67% (32/48) of ex-situ eggs.

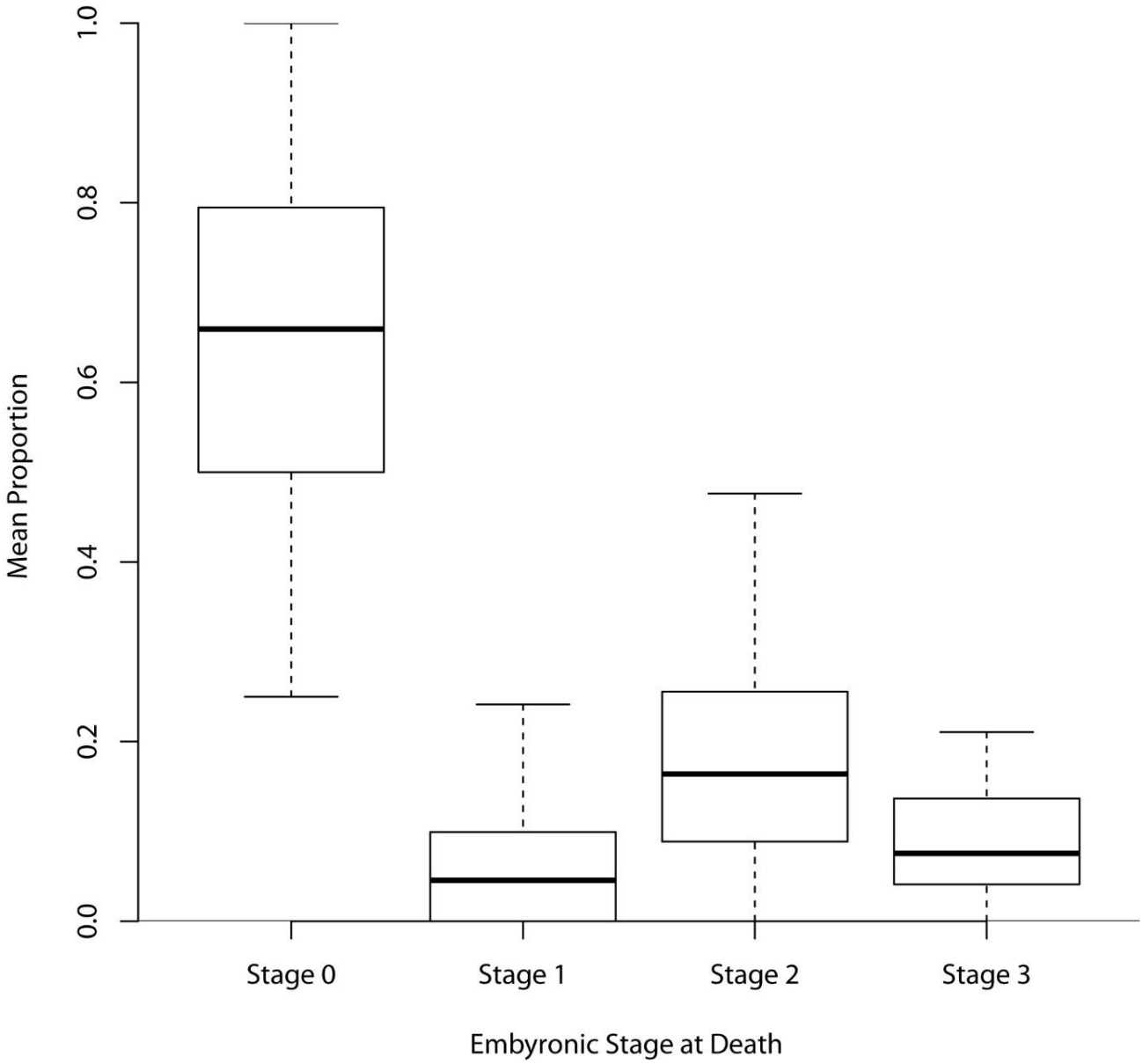


Figure 4.4. Average proportion of embryonic death at four stages of development in leatherback turtle nests. Nests (N = 20) were buried in a hatchery and had either normoxic (20.9% O_2; n = 10), hyperoxic (42% O_2; n = 10) gas pumped into the nest for the first five days after burial. Nests were excavated two days after emergence of hatchlings from the nest and embryos were staged according to Leslie et al.'s (1996) four-stage developmental chronology. Boxplot centre lines show medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

4.5 Discussion

Our results show that leatherback turtle hatching success progressively declined the longer that pre-ovipositional embryonic arrest was maintained. Although, incubation of eggs in hypoxia for three days did not significantly reduce hatching success. However, this could be a type II error, since the apparent difference in hatching success (72% hatching success after normoxic incubation versus 32% after 3 days of hypoxia) was large. Furthermore, five days of hypoxic incubation did lead to a significant reduction in hatching success (72% after normoxic incubation versus 4% after 5 days of hypoxia). Importantly, this suggests that extended periods of pre-ovipositional embryonic arrest, such as would be caused by a mother delaying nesting, could reduce her reproductive output. We propose that embryos are therefore temporally limited in their capacity to remain developmentally arrested, a phenomenon we dub the 'stale-egg effect'. Our findings corroborate those of another similar study conducted on sea turtles, in which it was found that hatching success was reduced by 65% after pre-ovipositional arrest was extended for five days in flatback turtles (Rings *et al.* 2015). In contrast, a three-day extension of arrest in green sea turtles did not result in reduced hatching success (Williamson *et al.* 2017). Importantly, our findings also contrast with those from an observational study of the Eastern Pacific leatherback turtle population which found a small but significant increase in hatching success with increasing intervals of up to 12 days between clutches (Rafferty *et al.* 2011). The same study demonstrated a higher proportion of early-stage embryonic death with increased inter-nesting intervals, suggesting that different factors act on early-stage and late-stage embryos to cause their mortality. Taken together, these observations indicate that the duration of pre-ovipositional embryonic arrest can have important reproductive and ecological ramifications for turtles.

4.5.1 Embryonic arrest and subsequent development

We found that white spot formation and hatching were delayed when eggs were placed in hypoxia, with the delay roughly equivalent to the time spent in hypoxia. This indicates that hypoxic incubation of eggs after oviposition maintains pre-ovipositional embryonic arrest in leatherback turtles, as it does in other species of turtle (Kennett *et al.* 1993; Rafferty *et al.* 2013; Rings *et al.* 2015; Williamson *et al.* 2017). Furthermore, white spot formation was not necessarily confirmation that individual eggs would develop successfully through to hatching, because almost all eggs formed white spots even though there was a reduced hatching success and higher early-stage mortality in the hypoxic treatments. Most embryos deemed to have died from the hypoxic treatments clearly had not developed past the stage of attachment to the eggshell when the embryonic development was monitored via candling. This provides further justification for monitoring white spot formation as a non-invasive method for estimating the fertilisation rate for a clutch of eggs (Abella *et al.* 2017), because we found that the white spot still formed even when embryos died at early developmental stages. Our finding also support the proposition that formation of the white spot may not always require an actively developing embryo and could depend upon a passive hydraulic response, such as drying of the shell and water uptake by the albumin when the egg moves from the oviduct to the nest (Warner *et al.* 2011; Williamson *et al.* 2017). Increased oxygen availability is clearly the trigger that causes embryonic development to recommence, but formation and growth of a white spot does not necessarily indicate that development will be successful in a normoxic environment.

Whilst we found that white spot formation might not always indicate that development has commenced, formation of the white spot is typically the first external sign that embryonic development has commenced after oviposition and usually occurs within one to five days in

marine turtles (Blanck and Sawyer 1981; Miller 1985; Thompson 1985; Chan 1989; Rafferty and Reina 2014; Abella *et al.* 2017). Our finding that leatherback turtle eggs took around four days to form white spots, once they were in aerobic conditions, is consistent with the finding of a previous study that leatherback eggs take between four and five days to form white spots, regardless of incubation temperature (Chan 1989). Egg incubation in turtles is influenced by temperature, and an incubation duration of 60-63 days for our ex-situ eggs that were incubated at a 30 ± 1ºC, is in reasonable agreement with previous investigations of the duration of leatherback turtle incubation (Miller 1982, 1985; Chan 1989; Thompson 1993).

None of our ex-situ experimental treatments had any impact upon hatchling fitness or morphology. Similarly, there was no detectable impact upon fitness of olive ridley hatchlings that were incubated in hypoxia for three days (Williamson, unpublished data). However, flatback turtle hatchlings were significantly larger when pre-ovipositional arrest was extended for five days (Rings *et al.* 2015). The same study also demonstrated that flatback turtles swim slower and crawl faster when incubated in hyperoxia for five days. Hatchling size, crawling and swimming abilities are important to the chances of hatchlings to survive and they may also influence dispersal patterns (Booth *et al.* 2013; Read *et al.* 2013; Sim *et al.* 2014; Rings *et al.* 2015). However, variation in oxygen availability during the first five days of incubation appears to have no detectable impact on leatherback turtle hatchling morphology or fitness traits.

Pre-ovipositional embryonic arrest provides leatherback mothers with some flexibility in the timing of nesting. However, delaying nesting and thereby extending arrest, because of adverse conditions or the presence of humans or predators, could have important ecological implications. Extreme storms or rain are examples of adverse conditions that can lead to delayed nesting (Plotkin *et al.* 1997). Large dry fronts of sand on nesting beaches could also increase the number

of failed nesting attempts and increase inter-nesting intervals. Our findings suggest that such instances of delayed nesting likely result in reduced hatching success. Therefore, there appears to be an ecological trade-off between the flexibility to adjust timing of nesting in response to adverse conditions and reduced reproductive output.

4.5.2 Hyperoxia and other potential causes of embryonic death

Exposure of leatherback turtle eggs to hyperoxia during the first five days of incubation did not improve hatching success or reduce early stage embryonic death, at least under our experimental conditions. Therefore, a lack of sufficient oxygen availability after oviposition does not appear to contribute to the low hatching success experienced in leatherback turtles. Clutch fertilisation rate does not appear to be a contributing factor (Bell *et al.* 2003). Oxygen availability and temperature within nests at Playa Grande (Costa Rica) do not appear to directly correlate with hatching success, even though there is spatial variation in hatching success within the nest (Wallace *et al.* 2004; Ralph *et al.* 2005). However, the opposite relationship was found for the St. Croix nesting population, with a correlation between nest minimum PO_2, maximum PCO_2 and temperature, and decreased hatching success, although this was primarily caused by increased late-stage embryonic death (Garrett *et al.* 2010), indicating that failure to break embryonic arrest was not the cause of mortality. High temperatures during early development have been shown to correlate with increased early-stage death in Costa Rica (Santidrián Tomillo *et al.* 2009). Bacterial or fungal infection has been suggested as a potential cause (Bell *et al.* 2003), but a study of the Columbian nesting population found no impact of microorganisms on hatching success (Patino-Martinez *et al.* 2012).

There are other possible causes for the high incidence of early-stage embryonic mortality in leatherbacks and there could be compounding interactions between all of these factors. It has been shown that within the Playa Grande population some mothers are better than others, as hatching success varied significantly with maternal identity (Bell *et al.* 2003; Rafferty *et al.* 2011). The underlying cause of the between-individual variation remains unknown, but could be due to differences in maternal physiology that cause their eggs to vary in their susceptibility to embryonic death. Maternal health indicators such as gamma globulin protein and red blood cell count correlated with hatching success in the Florida nesting population (Perrault *et al.* 2012). The same study also found that certain biochemical parameters correlated with hatching success, such as alkaline phosphatase activity, blood urea nitrogen, calcium, calcium:phosphorus ratio, carbon dioxide, cholesterol, creatinine, and phosphorus (Perrault *et al.* 2012). Variation in individual fecundity and the resources allocated to eggs could be impacted by variations in maternal health caused by differences in nesting migration interval and duration (Hewavisenthi and Parmenter 2002; Rafferty *et al.* 2011). However, the St. Croix population appears to exhibit no correlation between maternal identity and hatching success, with greater variation between different clutches from individual females (Garrett *et al.* 2010). It has also been shown that environmental contaminants accumulated by females may negatively impact hatching success (Guirlet *et al.* 2008; Guirlet *et al.* 2010; van de Merwe *et al.* 2010; Perrault *et al.* 2011; Stewart *et al.* 2011; De Andrés *et al.* 2016).

It is evident that there are many possible causes for low hatching success in leatherback turtle nests. Furthermore, it is likely that no cause is singularly responsible and that the various effects are compounding. The selection pressures that might permit the persistently low hatching success in leatherback turtles remain puzzling. Currently, we can only speculate about the

reasons why leatherback eggs are consistently less developmentally successful at hatching than those of other species of sea turtles. There is also little information about the historical hatching success of leatherback turtle populations. One hypothesis in favour of selection for reduced hatching success would be that it allows greater nest cavity space and potentially increased nest moisture levels for each hatchling produced. Extreme desiccation stress could possibly be buffered by the albumin of dead eggs that are adjacent to eggs that are still developing in the nest. However, the specific mechanisms and causes of the high incidence of embryonic death (especially at an early-stage) are still unclear (Bell *et al.* 2003; Garrett *et al.* 2010; Rafferty *et al.* 2011) and warrant further investigation.

4.5.3 Impact of incubation medium and nest size on hatching success

We found that eggs maintained in incubators had a greater hatching success than those buried in nests in the hatchery. A similar difference between eggs in a hatchery and those in an incubator was found for the Malaysian leatherback population (Chan 1989). Nests relocated to hatcheries have also been shown to have lower hatching success than natural nests incubated *in situ* (Eckert and Eckert 1990; Garcia *et al.* 2003; Pintus *et al.* 2009; Garrett *et al.* 2010; Sieg *et al.* 2011). However, this is not always the case and at some sites relocated nests have equal or greater hatching success than those left on the beach at the site of oviposition (Wyneken *et al.* 1988; Tuttle and Rostal 2010). Differences in overall hatching success between our hatchery and incubator experiments could be due to a reduced impact of microorganisms and metabolic heating on embryonic development and more stable respiratory gas concentrations in the incubators when compared to nests.

We also found that when only half a clutch of eggs was buried into a hatchery nest cavity, the time until emergence of hatchlings was significantly longer (3 days) than from whole clutches (two half-clutches combined). This is probably due to reduced metabolic heating of smaller nests (Godfrey *et al.* 1997; Wallace *et al.* 2004; Ralph *et al.* 2005) and increased effort and time required by smaller groups of hatchlings to escape the nest (Rusli *et al.* 2016). There was also trend for reduced hatching success (by 25%) in the half-sized nests compared to whole ones. However, this apparent effect of nest size was not statistically significant, so a larger sample size would be required to determine whether it is real or not. This trend also contrasts with the findings of a previous study, where halving clutches into two separate nests increased hatching success at a Malaysian nesting sites (Balasingam 1967). The cost of using incubators on a large-scale would be restrictive and relocation of clutches to hatcheries or to incubators should, where possible, be restricted to clutches that are likely to be doomed if left in place.

4.5.4 Conclusion

In summary, we found that the development of leatherback turtle embryos is arrested by hypoxia as it is in other species and that hypoxic incubation after oviposition can be used to extend arrest. However, extended pre-ovipositional embryonic arrest had negative impacts upon hatching success. We were able to administer hyperoxic gas to nests in-situ, but, incubation of leatherback turtle eggs in hyperoxic conditions did not improve hatching success or reduce the proportion of embryos that died at early stages of development. We also found that use of incubators reduced embryonic mortality when compared with eggs placed into in-situ hatchery nests. Our findings suggest that conditions that result in delayed nesting, such as increased intra- and inter-specific interactions, adverse abiotic conditions and increased nesting density at nesting beaches, could

have important ecological implications for reproductive output and recruitment for turtle populations.

4.6 Acknowledgements

This study could not have been completed without the help of Carlos Fernandez and from the staff at Reserva Pacuare and Ecology Project International. Particular thanks to Renato Bruno, Ashleigh Bandimere, Nerine Constant, Nathan Goldschmidt, Tarant Hill, Lucy McNaught and Adam Skorulis for assistance in the field and help with the incubators. Gracias a Germaine Barrantes, Pedro Morales Chávez y Hyeily Hernández González por toda su ayuda. We would like to express our gratitude to Stanley Rodriguez, Stamatina Skliros and everyone at the Estacion Las Tortugas for help with this study. Thank you to Paul Cooper and the staff at the workshop of the Australian National University (ANU) for assistance with construction of the timed-valves for the hatchery gas experiment. Finally, thanks to Anthony Rafferty, for inspiration and encouragement for this particular study.

4.7 References

Abella, E, García-Cerdá, R, Marco, A (2017) Non-invasive monitoring of the fertilization rate in sea turtle nests: comparison between two techniques. *Basic and Applied Herpetology* **31**, in press.

Balasingam, E (1967) The ecology and conservation of the leathery turtle *Dermochelys coriacea* (Linn.) in Malaya. *Micronesica* **3**, 37-43.

Bell, BA, Spotila, JR, Paladino, FV, Reina, RD (2003) Low reproductive success of leatherback turtles, *Dermochelys coriacea*, is due to high embryonic mortality. *Biological Conservation* **115**, 131-138.

Bilinski, JJ, Reina, RD, Spotila, JR, Paladino, FV (2001) The effects of nest environment on calcium mobilization by leatherback turtle embryos (*Dermochelys coriacea*) during development. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **130**, 151-162.

Blanck, CE, Sawyer, RH (1981) Hatchery practices in relation to early embryology of the loggerhead sea turtle, *Caretta caretta* (Linné). *Journal of Experimental Marine Biology and Ecology* **49**, 163-177.

Booth, DT, Feeney, R, Shibata, Y (2013) Nest and maternal origin can influence morphology and locomotor performance of hatchling green turtles (*Chelonia mydas*) incubated in field nests. *Marine Biology* **160**, 127-137.

Boulon, RH, Dutton, PH, McDonald, DL (1988) Leatherback turtles (*Dermochelys coriacea*) on St. Croix, U.S. Virgin Islands: Fifteen years of conservation. *Chelonian Conservation & Biology* **2**,

Chan, E-H (1989) White spot development, incubation and hatching success of leatherback turtle (*Dermochelys coriacea*) eggs from Rantau Abang, Malaysia. *Copeia* **1989**, 42-47.

Chan, E-H, Liew, H-C (1996) Decline of the leatherback population in Terengganu, Malaysia, 1956-1995. *Chelonian Conservation & Biology* **2**, 196-203.

Chan, E-H, Salleh, HU, Liew, H-C (1985) Effects of handling on hatchability of eggs of the leatherback turtle, *Dermochelys coriacea*. *Pertanika* **8**, 265-271.

De Andrés, E, Gómara, B, González-Paredes, D, Ruiz-Martín, J, Marco, A (2016) Persistent organic pollutant levels in eggs of leatherback turtles (*Dermochelys coriacea*) point to a decrease in hatching success. *Chemosphere* **146**, 354-361.

Dornfeld, TC, Robinson, NJ, Tomillo, PS, Paladino, FV (2015) Ecology of solitary nesting olive ridley sea turtles at Playa Grande, Costa Rica. *Marine Biology* **162**, 123-139.

Dutton, DL, Dutton, PH, Chaloupka, M, Boulon, RH (2005) Increase of a Caribbean leatherback turtle *Dermochelys coriacea* nesting population linked to long-term nest protection. *Biological Conservation* **126**, 186-194.

Eckert, KL, Eckert, SA (1990) Embryo mortality and hatch success in *In situ* and translocated leatherback sea turtle *Dermochelys coriacea* eggs. *Biological Conservation* **53**, 37-46.

Eckert, KL, Wallace, BP, Frazier, JG, Eckert, SA, Pritchard, PCH, 2012. Synopsis of the biological data on the leatherback sea turtle (*Dermochelys coriacea*). U.S. Department of Interior, Fish and Wildlife Service, Washington, D.C.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Fordham, DA, Georges, A, Corey, B (2007) Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise. *Aquaculture* **270**, 105-114.

Garcia, A, Ceballos, G, Adaya, R (2003) Intensive beach management as an improved sea turtle conservation strategy in Mexico. *Biological Conservation* **111**, 253-261.

Garrett, K, Wallace, BP, Garner, J, Paladino, FV (2010) Variations in leatherback turtle nest environments: Consequences for hatching success. *Endangered Species Research* **11**, 147-155.

Godfrey, MH, Barreto, R, Mrosovsky, N (1997) Metabolically-generated heat of developing eggs and its potential effect on sex ratio of sea turtle hatchlings. *Journal of Herpetology* **31**, 616-619.

Guirlet, E, Das, K, Girondot, M (2008) Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea*) of French Guiana. *Aquatic Toxicology* **88**, 267-276.

Guirlet, E, Das, K, Thomé, JP, Girondot, M (2010) Maternal transfer of chlorinated contaminants in the leatherback turtles, *Dermochelys coriacea*, nesting in French Guiana. *Chemosphere* **79**, 720-726.

Hewavisenthi, S, Parmenter, CJ (2002) Incubation environment and nest success of the flatback turtle (*Natator depressus*) from a natural nesting beach. *Copeia* **2002**, 302-312.

Hughes, GR (1996) Nesting of leatherback turtle (*Dermochelys coriacea*) in Tongaland, KwaZulu-Natal, South Africa, 1963-1996. *Chelonian Conservation & Biology* **2**, 153-158.

Kennett, R, Georges, A, Palmerallen, M (1993) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Leslie, AJ, Penick, DN, Spotila, JR, Paladino, FV (1996) Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990-1991. *Chelonian Conservation & Biology* **2**, 159-168.

Licht, P (1980) Evolutionary and functional aspects of pituitary gonadotropins in the green turtle, *Chelonia mydas*. *American Zoologist* **20**, 565-574.

Licht, P (1982) Endocrine patterns in the reproductive cycle of turtles. *Herpetologica* **38**, 51-61.

Licht, P, Owens, DW, Cliffton, K, Penaflores, C (1982) Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, *Lepidochelys olivacea*. *General and Comparative Endocrinology* **48**, 247-253.

Lynn, WG, von Brand, T (1945) Studies on the oxygen consumption and water metabolism of turtle embryos. *The Biological Bulletin* **88**, 112-125.

Miller, JD (1982) Development of marine turtles. University of New England.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Miller, JD (1997) Reproduction in sea turtles. In 'The biology of sea turtles.' (Eds PL Lutz, JA Musick.) (CRC Press: Boca Raton, Florida)

Owens, D, Morris, YA (1985) The comparative endocrinology of sea turtles. *Copeia* **1985**, 723-735.

Patino-Martinez, J, Marco, A, Quiñones, L, Abella, E, Abad, RM, Diéguez-Uribeondo, J (2012) How do hatcheries influence embryonic development of sea turtle eggs? Experimental analysis and isolation of microorganisms in leatherback turtle eggs. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **317A**, 47-54.

Perrault, J, Wyneken, J, Thompson, LJ, Johnson, C, Miller, DL (2011) Why are hatching and emergence success low? Mercury and selenium concentrations in nesting leatherback sea turtles (*Dermochelys coriacea*) and their young in Florida. *Marine Pollution Bulletin* **62**, 1671-1682.

Perrault, JR, Miller, DL, Eads, E, Johnson, C, Merrill, A, Thompson, LJ, Wyneken, J (2012) Maternal health status correlates with nest success of leatherback sea turtles (*Dermochelys coriacea*) from Florida. *PLoS One* **7**, e31841.

Pike, DA (2008) Natural beaches confer fitness benefits to nesting marine turtles. *Biology Letters* **4**, 704-706.

Pintus, KJ, Godley, BJ, McGowan, A, Broderick, AC (2009) Impact of clutch relocation on green turtle offspring. *Journal of Wildlife Management* **73**, 1151-1157.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

R Core Team (2016) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Reina, RD (2014) The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. *Journal of Experimental Marine Biology and Ecology* **450**, 91-97.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**, e21038.

Ralph, CR, Reina, RD, Wallace, BP, Sotherland, PR, Spotila, JR, Paladino, FV (2005) Effect of egg location and respiratory gas concentrations on developmental success in nests of the leatherback turtle, *Dermochelys coriacea*. *Australian Journal of Zoology* **53**, 289-294.

Read, T, Booth, DT, Limpus, CJ (2013) Effect of nest temperature on hatchling phenotype of loggerhead turtles (*Caretta caretta*) from two South Pacific rookeries, Mon Repos and La Roche Percée. *Australian Journal of Zoology* **60**, 402-411.

Reina, RD, Mayor, PA, Spotila, JR, Piedra, R, Paladino, FV (2002) Nesting ecology of the leatherback turtle, *Dermochelys coriacea*, at Parque Nacional Marino Las Baulas, Costa Rica: 1988-1989 to 1999-2000. *Copeia* **2002**, 653-664.

Reina, RD, Spotila, JR, Paladino, FV, Dunham, AE (2009) Changed reproductive schedule of eastern Pacific leatherback turtles *Dermochelys coriacea* following the 1997–98 El Niño to La Niña transition. *Endangered Species Research* **7**, 155-161.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Risley, P (1944) Arrested development in turtle embryos. *The Anatomical Record* **88**, 454-455.

Rivalan, P, Prévot-Julliard, A-C, Choquet, R, Pradel, R, Jacquemin, B, Girondot, M (2005) Trade-off between current reproductive effort and delay to next reproduction in the leatherback sea turtle. *Oecologia* **145**, 564-574.

Rivas, ML, Fernández, C, Marco, A (2016) Nesting ecology and population trend of leatherback turtles *Dermochelys coriacea* at Pacuare Nature Reserve, Costa Rica. *Oryx* **50**, 274-282.

Rostal, DC, Grumbles, JS, Palmer, KS, Lance, VA, Spotila, JR, Paladino, FV (2001) Changes in gonadal and adrenal steroid levels in the leatherback sea turtle (*Dermochelys coriacea*) during the nesting cycle. *General and Comparative Endocrinology* **122**, 139-147.

Rostal, DC, Paladino, FV, Patterson, RM, Spotila, JR (1996) Reproductive physiology of nesting leatherback turtles (*Dermochelys coriacea*) at Las Baulas National Park, Costa Rica. *Chelonian Conservation & Biology* **2**, 230-236.

Rusli, MU, Booth, DT, Joseph, J (2016) Synchronous activity lowers the energetic cost of nest escape for sea turtle hatchlings. *Journal of Experimental Biology* **219**, 1505-1513.

Saba, VS, Santidrián Tomillo, P, Reina, RD, Spotila, JR, Musick, JA, Evans, DA, Paladino, FV (2007) The effect of the El Niño Southern Oscillation on the reproductive frequency of eastern Pacific leatherback turtles. *Journal of Applied Ecology* **44**, 395-404.

Santidrián Tomillo, P, Saba, VS, Blanco, GS, Stock, CA, Paladino, FV, Spotila, JR (2012) Climate driven egg and hatchling mortality threatens survival of eastern Pacific leatherback turtles. *PLoS One* **7**,

Santidrián Tomillo, P, Saba, VS, Piedra, R, Paladino, FV, Spotila, JR (2008) Effects of illegal harvest of eggs on the population decline of leatherback turtles in Las Baulas Marine National Park, Costa Rica. *Conservation Biology* **22**, 1216-1224.

Santidrián Tomillo, P, Suss, JS, Wallace, BP, Magrini, KD, Blanco, G, Paladino, FV, Spotila, JR (2009) Influence of emergence success on the annual reproductive output of leatherback turtles. *Marine Biology* **156**, 2021-2031.

Santidrián Tomillo, P, Vélez, E, Reina, RD, Piedra, R, Paladino, FV, Spotila, JR (2007) Reassessment of the leatherback turtle (*Dermochelys coriacea*) nesting population at Parque Nacional Marino Las Baulas, Costa Rica: Effects of conservation efforts. *Chelonian Conservation & Biology* **6**, 54-62.

Sieg, AE, Binckley, CA, Wallace, BP, Tomillo, PS, Reina, RD, Paladino, FV, Spotila, JR (2011) Sex ratios of leatherback turtles: Hatchery translocation decreases metabolic heating and female bias. *Endangered Species Research* **15**, 195-204.

Sim, EL, Booth, DT, Limpus, CJ, Guinea, ML (2014) A comparison of hatchling locomotor performance and scute pattern variation between two rookeries of the flatback turtle (*Natator depressus*). *Copeia* **2014**, 339-344.

Spotila, JR, Reina, RD, Steyermark, AC, Plotkin, PT, Paladino, FV (2000) Pacific leatherback turtles face extinction. *Nature* **405**, 529-30.

Stewart, KR, Keller, JM, Templeton, R, Kucklick, JR, Johnson, C (2011) Monitoring persistent organic pollutants in leatherback turtles (*Dermochelys coriacea*) confirms maternal transfer. *Marine Pollution Bulletin* **62**, 1396-1409.

Tapilatu, RF, Dutton, PH, Tiwari, M, Wibbels, T, Ferdinandus, HV, Iwanggin, WG, Nugroho, BH (2013) Long-term decline of the western Pacific leatherback, *Dermochelys coriacea*: a globally important sea turtle population. *Ecosphere* **4**, 1-15.

Thompson, MB (1985) Functional significance of the opaque white patch in eggs of *Emydura macquarii*. In 'Biology of Australasian frogs and reptiles.' (Eds G Grigg, R Shine, H Ehmann.) pp. 387-395. (Surrey Beatty in association with The Royal Zoological Society of New South Wales: Chipping Norton, NSW)

Thompson, MB (1993) Oxygen consumption and energetics of development in eggs of the leatherback turtle, *Dermochelys coriacea*. *Comparative Biochemistry and Physiology - A Physiology* **104**, 449-453.

Tucker, AD, Frazer, NB (1991) Reproductive variation in leatherback turtles, *Dermochelys coriacea*, at Culebra National Wildlife Refuge, Puerto Rico. *Herpetologica* **47**, 115-124.

Tuttle, J, Rostal, D (2010) Effects of nest relocation on nest temperature and embryonic development of loggerhead sea turtles (*Caretta caretta*). *Chelonian Conservation and Biology* **9**, 1-7.

Valverde, RA (1996) Corticosteroid dynamics in a free-ranging population of olive ridley sea turtles (*Lepidochelys olivacea* Eschscholtz, 1829) at Playa Nancite, Costa Rica as a function of their reproductive behaviour. Texas A&M University.

van de Merwe, JP, Hodge, M, Whittier, JM, Ibrahim, K, Lee, SY (2010) Persistent organic pollutants in the green sea turtle *Chelonia mydas*: nesting population variation, maternal transfer, and effects on development. *Marine Ecology Progress Series* **403**, 269-278.

Wallace, BP, DiMatteo, AD, Bolten, AB, Chaloupka, MY, Hutchinson, BJ, Abreu-Grobois, FA, Mortimer, JA, Seminoff, JA, Amorocho, D, Bjorndal, KA, Bourjea, J, Bowen, BW, Briseño Dueñas, R, Casale, P, Choudhury, BC, Costa, A, Dutton, PH, Fallabrino, A, Finkbeiner, EM, Girard, A, Girondot, M, Hamann, M, Hurley, BJ, López-Mendilaharsu, M, Marcovaldi, MA, Musick, JA, Nel, R, Pilcher, NJ, Troëng, S, Witherington, B, Mast, RB (2011) Global conservation priorities for marine turtles. *PLoS One* **6**, e24510.

Wallace, Bryan P, Sotherland, Paul R, Spotila, James R, Reina, Richard D, Franks, Bryan F, Paladino, Frank V (2004) Biotic and abiotic factors affect the nest environment of embryonic leatherback turtles, *Dermochelys coriacea*. *Physiological and Biochemical Zoology* **77**, 423-432.

Wallace, BP, Tiwari, M, Girondot, M (2013) *Dermochelys coriacea*. *The IUCN Red List of Threatened Species* **2013**,

Warner, DA, Moody, MA, Telemeco, RS (2011) Is water uptake by reptilian eggs regulated by physiological processes of embryos or a passive hydraulic response to developmental

environments? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **160**, 421-425.

Whitmore, CP, Dutton, PH (1985) Infertility, embryonic mortality and nest-site selection in leatherback and green sea turtles in Suriname. *Biological Conservation* **34**, 251-272.

Williamson, SA, Evans, RG, Reina, RD (2017) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Wyneken, J, Burke, TJ, Salmon, M, Pedersen, DK (1988) Egg failure in natural and relocated sea turtle nests. *Journal of Herpetology* **22**, 88-96.

Chapter 5. Synchronised nesting aggregations (arribadas) are associated with enhanced capacity for extended embryonic arrest in olive ridley sea turtles.

Sean A. Williamson[1], Roger G. Evans[2], Nathan Robinson[3, 4, 5] and Richard D. Reina[1]

[1] School of Biological Sciences, Monash University, Clayton, Victoria, Australia

[2] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton, Victoria, Australia

[3] The Leatherback Trust, Goldring-Gund Marine Biology Station, Playa Grande, Costa Rica

[4] Department of Biology, Indiana-Purdue University, Fort Wayne, Indiana, USA

[5] Present address: Cape Eleuthera Institute, Eleuthera, The Bahamas

This manuscript is currently being prepared for submission to a peer-reviewed journal.

5.1 Abstract

Aggregated and synchronised nesting is an unusual and interesting evolutionary life-history tactic exhibited by two species of sea turtle from the *Lepidochelys* genus. Aggregated nesting events, termed 'arribadas', can involve hundreds of thousands of females congregating at a single nesting beach over a few days and nights to oviposit their eggs. However, individuals of both species of *Lepidochelys* show behavioural polymorphism and can also nest solitarily, in non-arribada events. The two tactics are associated with different inter-nesting intervals of 14 and 28 days for non-arribada and arribada nesters, respectively. Consequently, embryos are maintained in pre-ovipositional embryonic arrest in the hypoxic oviduct for different lengths of time depending on the tactic used. Here, we investigated whether embryos that are oviposited during an arribada differ in their capacity to be maintained in pre-ovipositional arrest when compared with those oviposited during a non-arribada event. Olive ridley sea turtle (*Lepidochelys olivacea*) eggs from eight clutches (four from each nesting tactic) were divided among seven treatments after oviposition; normoxia (control; 21% O_2), or hypoxia (1% O_2) for 3, 3.5, 4, 8, 15 or 30 days, before being all returned to normoxia. Arribada eggs generally had lower hatching success (<25%) across all treatments, whereas non-arribada eggs exhibited much greater hatching success in the control and 3-day hypoxia treatment (>65%) than in the longer hypoxia treatments. However, arribada eggs were capable of extending pre-ovipositional arrest for longer, with some eggs from the 8- and 15-day hypoxia treatment still hatching while no non-arribada eggs hatched after more than four days in hypoxia. This difference in embryonic capacity to survive extended periods of arrest may be an important mechanism facilitating the arribada behaviour by allowing longer inter-nesting intervals. Our finding provides an intriguing

insight into the physiological mechanisms that are integral to this unique mass-nesting behaviour.

Keywords: *Embryonic Development, Hypoxia, Delayed Nesting, Synchronised Nesting, Lepidochelys olivacea*

5.2 Introduction

The evolution of synchronised reproductive behaviour has been documented in a range of animals and plants, but aggregated reproductive synchrony is unusual amongst large vertebrates (reviewed in Bernardo and Plotkin 2007). Two well-known exceptions are those of the *Lepidochelys* sea turtle genus, which nest in large synchronised aggregations termed 'arribadas'. However, both species of *Lepidochelys* (olive ridley sea turtle *Lepidochelys olivacea* and Kemp's ridley sea turtle *Lepidochelys kempii*) display behavioural reproductive polymorphism, whereby individuals can interchangeably nest in arribadas or nest solitarily in individual nesting events as other species of sea turtle do (Hirth 1980; Kalb 1999; Bernardo and Plotkin 2007). Arribadas can involve hundreds of thousands of turtles nesting at a single nesting beach usually over three to four days and nights (Valverde *et al.* 1998; Bernardo and Plotkin 2007).

A few explanations regarding the selective advantages for arribada nesting behaviour have been proposed. Nesting in large aggregations has been suggested to improve mate-finding ability (Plotkin *et al.* 1996; Bernardo and Plotkin 2007) in a predominately widespread pelagic species (Plotkin *et al.* 1995). Synchronised reproductive behaviour also offers potential fitness benefits through higher rates of multiple mating and paternity (Plotkin *et al.* 1997; Jensen *et al.* 2006). Arribadas have also been suggested as a mechanism to saturate predators (Pritchard 1969; Cornelius *et al.* 1991; Eckrich and Owens 1995) in order to increase hatchling survival rate,

including during early hatchling dispersal. Potential cues that stimulate arribada aggregations include meteorological (wind and rain), lunar, social, and sensory (olfaction) cues that have been suggested as potential triggers for nesting (Bernardo and Plotkin 2007). However, the proximate and ultimate causes remain to be determined.

The behavioural nesting polymorphism is associated with different inter-nesting intervals (the period between subsequent clutches). Olive ridley sea turtles generally oviposit two clutches 14 days apart when nesting solitarily, or 28 days apart when nesting in an arribada (Pritchard 1969; Plotkin *et al.* 1997; Kalb 1999; Rostal 2007). Follicles for the subsequent clutch are ovulated one to two days after the first nest is oviposited (Licht 1982; Licht *et al.* 1982; Rostal 2007). The ova are fertilised shortly after ovulation (Solomon and Baird 1979; Owens 1980) and embryos have commenced development within three days of the first nesting (Miller 1982, 1985). Prior to oviposition all turtle embryos arrest development at the gastrulation stage of development (Miller 1982; Ewert 1985; Miller 1985) and this pre-ovipositional embryonic arrest is maintained by hypoxia (low oxygen) in the oviducts, thereby affording the mother flexibility in her nesting date (Rafferty and Reina 2012; Rafferty *et al.* 2013). The arrest can be artificially extended by placing eggs in hypoxia within 12 hours after oviposition (Kennett *et al.* 1993; Rings *et al.* 2015; Williamson *et al.* 2017). To meet the schedule needed for synchronised nesting, ridley females nesting in arribadas are probably keeping embryos in arrest for 14 days longer than those nesting solitarily. Whether this putative longer period of pre-ovipositional embryonic arrest is associated with altered egg physiology, resulting in altered capacity for extended arrest, is the subject of this investigation.

We hypothesise that eggs oviposited in arribada and solitary events differ in their ability to be maintained in embryonic arrest after oviposition. It is known that turtle eggs are temporally

limited in their capacity to extend arrest (Kennett *et al.* 1993; Fordham *et al.* 2006; Rings *et al.* 2015). It is possible that arribada eggs have a greater ability to maintain arrest so that these females are able to nest at longer intervals. Alternatively, non-arribada eggs may be able to maintain arrest for longer under experimental conditions after they have been laid because they may have spent less time in pre-ovipositional arrest in the oviduct. Here, we assessed the capacity of arribada and non-arribada eggs to be maintained in pre-ovipositional embryonic arrest, by incubating eggs in hypoxia for varying lengths of time after oviposition.

5.3 Methods

5.3.1 Regulatory approval

Monash University's School of Biological Sciences Animal Ethics Committee (Approval BSCI/2015/10) approved all experimental procedures. Field research was carried out 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).

5.3.2 Egg collection

All egg clutches were collected from different nesting females at Playa Ostional in the Refugio de Vida Silvestre Ostional, Costa Rica. Between 9:09 and 9:36 pm on the 26th of October 2015, four clutches of eggs (n = 96 to 104 eggs per clutch, 399 eggs total) were collected from nesting females that were not nesting during an arribada. Then, between 4:03 and 4:14 pm on the 7th of November 2015, a further four clutches of eggs (n = 57 to 95 eggs per clutch, 308 eggs total) were collected from nesting females that were nesting during an arribada. All clutches were

collected by placing a plastic bag into the nesting cavity. The maximum time between oviposition of the first and last egg of each clutch was 18 minutes. Once the last egg was oviposited the bag with eggs inside was removed from the nest and quickly (< 5 mins) carried a short distance (< 1 km) to the MINAE station at Ostional. All eggs were individually numbered with soft pencil and allocated to one of seven treatments. Placement of eggs into their respective treatments was complete within 40 to 100 minutes after oviposition. Once all eggs were in their treatments they were driven in a car for approximately two and a half hours to the headquarters of Parque Nacional Marino Las Baulas for incubation.

5.3.3 Experimental design

Each clutch of eggs was evenly divided between seven treatments (Table 5.1). The first was a normoxic control, in which eggs were randomly assigned one of three incubators (described below), placed directly into sand and kept in normoxia (~21% O_2) for the duration of the experiment. The other six treatments involved placing eggs into ziplock bags (Ziploc, United States) as described previously (Chapter 3). Within each bag the eggs rested on a wire mesh above 10 mL of distilled water in a plastic container with no lid. There were between three and four separate ziplock bags and containers used per treatment. Nitrogen gas (100% industrial grade; INFRA G.I., San Jose, Costa Rica) was then passed through the bag at eight litres per minute for three minutes through in-flow and out-flow valves, that had been inserted at each end of the ziplock bag, before the valves were closed. Three times daily, for the duration of the experimental treatments, each bag was re-gassed as described above. The six hypoxic treatments lasted either 3, 3.5, 4, 8, 15, or 30 days. Following the completion of each treatment period eggs were removed from their bag and placed into sand in incubators (described below). When the eggs were removed from hypoxia a subsample of eggs (n = 2 to 4 per treatment, 18 eggs total)

were opened and staged according to Miller's (1985) 31-stage development chronology. A subsample of the control treatment eggs (n = 3) were also opened and staged within two hours of oviposition.

5.3.4 Incubation

Other than when eggs were kept in hypoxia they were incubated in normoxia in beach sand (7% moisture content by mass) within incubators (GQF HovaBator model 1632; Grandview Management, Baldivis, Australia) set to 28°C. Eggs were monitored three times daily for formation of the opaque white spot on the surface of the egg. Due to condensation whilst eggs were in the ziplock bags it was not possible to accurately monitor the timing of formation of the white spots on eggs. Presence or absence of a white spot was recorded when eggs were removed from hypoxia. Twenty days after eggs were removed from their respective treatments they were transported a short distance (< 200 m) to a beach hatchery and buried in nest cavities that had been dug by hand to mimic the natural shape of an olive ridley sea turtle nest.

Table 5.1. Comparison of embryonic development between treatments.

	Nesting tactic	Control	3-day	3.5-day	4-day	8-day	15-day	30-day
No. eggs	Non-arribada	71	53	55	54	56	54	56
	Arribada	48	44	44	43	44	43	42
No. eggs opened after treatment	Non-arribada	2	2	1	1	2	2	2
	Arribada	1	0	2	2	2	2	0
Embryonic stage of opened eggs*	Non-arribada	6 & 6	-	9	8	6 & 8	8 & 8	6 & 10
	Arribada	6	6 & 8	10 & 11	8 & 12	6 & 11	8 & 14	-

Olive ridley eggs were collected from non-arribada and arribada nesting females and incubated following different durations of post-oviposition hypoxia.
*Staged according to Miller's (1985) 31-stage developmental chronology.

5.3.5 Hatching and embryonic death

The first twenty hatchlings to emerge from each nest had their mass (± 0.1 g) recorded using a scale and their head width, carapace length and width (all ± 0.1 mm) measured using a digital calliper. Two days after first emergence of hatchlings each nest was excavated. Unhatched eggs were counted to determine the hatching success (proportion of eggs to hatch) for each treatment. The unhatched eggs were then opened and the stage of embryonic development was determined according to Leslie *et al.*'s (1996) field embryo-staging method. The method classifies Miller's (1985) 31-stage developmental chronology into four broader stages as summarised by Rafferty *et al.* (2011). We were mostly unable to identify the unique pencil marking on each eggshell, so were unable to determine the maternal identification for hatched and unhatched eggs.

5.3.6 Statistical analysis

To assess between-treatment variation in the proportion of eggs to form white spots, form white spots in hypoxia, hatch, and die at Leslie *et al.*'s (1996) four developmental stages, whilst adjusting for nesting strategy of the mother (arribada vs non-arribada) Cochran-Mantel-Haenszel (CMH) tests were used. Woolf's test was used to check for any two-way interaction between treatment and nesting strategy for the proportions described above. Post-hoc analysis of the CMH tests was conducted by assessing between-treatment variation using pair-wise Bonferroni corrected chi-squared tests. To assess within-treatment differences in hatching success between arribada and non-arribada eggs Fisher's exact tests were used. To assess between-treatment and between-nesting tactic variation in latency (time) till white spot, aerobic latency (total time excluding time spent in hypoxia), and hatchling morphometric traits (mass, head width, carapace length and width), two-way Analysis of variance (ANOVA) tests were used to. Post-hoc comparisons were made using Tukey's

Honest Significant Difference (HSD) test. The assumptions of homoscedasticity and normality for ANOVA models were assessed using quantile plots in the Plot package in R studio (R Core Team 2016). All analyses were conducted using R software (R Core Team 2016). Values presented are mean ± standard error unless otherwise stated. Two-tailed $p \leq 0.05$ was considered statistically significant.

5.4 Results

5.4.1 White spot formation

There was significant between-treatment variation in the proportion of eggs to form white spots (X^2_{CMH} = 61.46, d.f. = 6, $p < 0.0001$), with no significant interaction with nesting strategy (Woolf test X^2 = 0.003, d.f. = 1, p = 0.96). Fewer eggs in the 15-day hypoxia treatment formed white spots when compared to all other treatments except the 30-day hypoxia treatment (Table 5.2). A large proportion of eggs formed white spots whilst they were still in hypoxia (Table 5.2), and in one case a white spot formed on the underside of an egg. For eggs that formed white spots after removal from hypoxia, there was a significant interaction between treatment and nesting tactic in the latency (time) until white spot formation (interaction term: F = 4.39, d.f. = 2, p = 0.01; Table 5.3). However, latency until white spot formation generally increased with increasing time spent in hypoxia for both nesting tactics (Treatment effect: F = 3502, d.f. = 4, $p < 0.0001$; Table 5.3). After accounting for time spent in hypoxia, there was still a significant interaction between treatment and nesting tactic in the aerobic latency (total time excluding time in hypoxia) until white spot formation (interaction term: F = 4.39, d.f. = 2, p = 0.01; Table 5.3). Aerobic latency until white spot formation was shorter for arribada eggs in the 3-day and 4-day hypoxia treatments when compared with control (Table 5.3).

5.4.2 Hatching

Latency to first emergence of a hatchling from the nest varied among treatments, but the differences did not correspond directly with the duration of hypoxic incubation (Table 5.3). The proportion of eggs to hatch varied significantly between treatments (X^2_{CMH} = 169.62, d.f. = 6, $p < 0.0001$; Figure 5.1), with no interaction with nesting strategy (Woolf test X^2 = 0.03, d.f. = 1, p = 0.86). The general trend was for a reduction in hatching success when eggs were kept in hypoxia for longer periods of time (Figure 5.1). However, there were differences in hatching success between the arribada and the non-arribada eggs within each treatment (Fisher exact tests p always ≤ 0.05; Figure 5.1). Generally, the non-arribada eggs had greater hatching success than the arribada eggs within each treatment (Figure 5.1). However, after 8 or 15 days in hypoxia all non-arribada eggs failed to hatch, whereas the arribada eggs that were subjected to these two treatments had hatching success comparable to the arribada eggs from the other treatments (Figure 5.1). No eggs, from either nesting strategy, hatched after 30 days in hypoxia (Figure 5.1).

Table 5.2. Proportion of white spot formation between treatments.

	Nesting tactic	Control	3-day	3.5-day	4-day	8-day	15-day	30-day	*P*-value*
White spots (WS) formed	Non-arribada	69 (100%)	52 (100%)	54 (100%)	53 (100%)	53 (88.1%)	36 (69.2%)	55 (98.2%)	< 0.0001
	Arribada	46 (97.9%)	43 (97.7%)	41 (97.6%)	42 (100%)	43 (97.7%)	40 (97.6%)	33 (78.6%)	< 0.0001
WS formed once in normoxia	Non-arribada	69 (100%)	7 (13.5%)	0 (0%)	19 (35.8%)	24 (45.3%)	2 (5.6%)	0 (0%)	< 0.0001
	Arribada	46 (100%)	4 (9.3%)	0 (0%)	10 (23.8%)	0 (0%)	0 (0%)	0 (0%)	< 0.0001

Olive ridley eggs were collected from non-arribada and arribada nesting females and incubated following different durations of post-oviposition hypoxia.
*Chi-squared tests were used to assess between-treatment differences for separated arribada and non-arribada data.

Table 5.3. Latency to white spot (WS) formation and first hatchling emergence between treatments.

	Nesting tactic	Control	3-day	3.5-day	4-day	8-day	15-day	30-day
Mean latency to WS (d)	Non-arribada	$0.8 \pm 0.0^{A*}$	3.5 ± 0.1^{B}	-	4.8 ± 0.1^{C}	8.8 ± 0.1^{D}	16.0 ± 0^{E}	-
	Arribada	$1.0 \pm 0.0^{X*}$	3.4 ± 0.2^{Y}	-	4.6 ± 0.1^{Z}	-	-	-
Mean aerobic latency to WS (d)	Non-arribada	$0.8 \pm 0.4^{A*}$	0.5 ± 0.1^{A}	-	0.8 ± 0.1^{A}	0.8 ± 0.1^{A}	1.0 ± 0^{A}	-
	Arribada	$1.0 \pm 0.0^{X*}$	0.4 ± 0.2^{Y}	-	0.6 ± 0.1^{Y}	-	-	-
Latency to first hatchling emergence (d)	Non-arribada	49	52	53	52	-	-	-
	Arribada	50	52	51	51	54	57	-

Olive ridley eggs were collected from non-arribada and arribada nesting females and incubated following different durations of post-oviposition hypoxia. All eggs from the 3-day and 30-day treatment formed WSs while in hypoxia, as did the 8- and 15-day arribada eggs, so there are no data for latency to white spot. Furthermore, no 30-day eggs hatched, neither did any 8- and 15-day non-arribada eggs, so there are no data for latency to hatchling emergence. When superscript letters are the same, there was no significant between-group difference within nesting tactic according to an ANOVA and Tukey's HSD post-hoc test. An asterisk (*) denotes a significant difference within each treatment between each nesting tactic according to an ANOVA and Tukey's HSD post-hoc test.

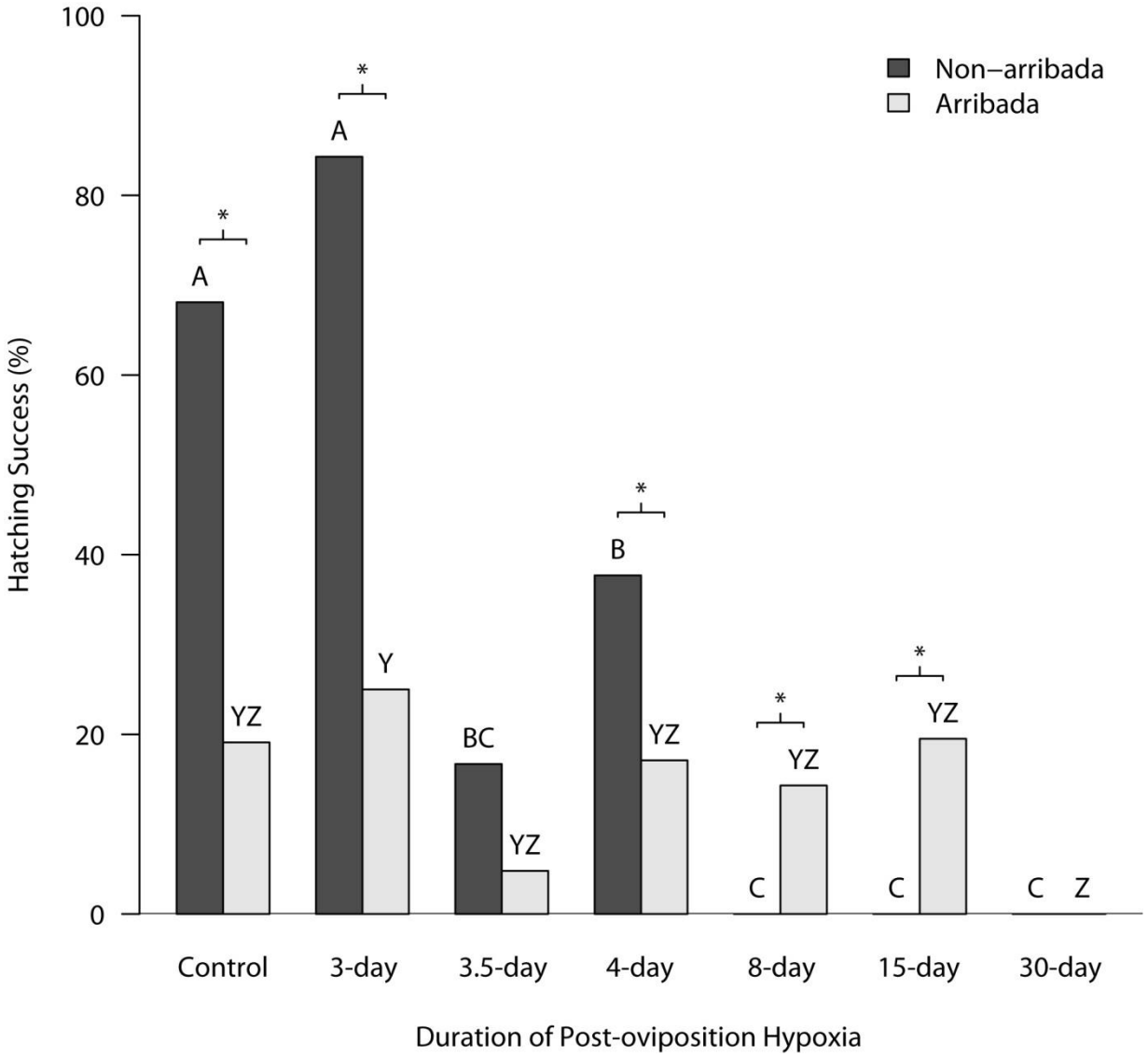


Figure 5.1. Olive ridley hatching success (%) between various treatments, partitioned according to maternal nesting tactic. Eggs (*N* = 686) were collected from either non-arribada (*N* = 387) or arribada nesting events (*N* = 299) and placed in either normoxia (21% O_2; control), or hypoxia (1% O_2) for 3, 3.5, 4, 8, 15, or 30 days. After their respective treatments all eggs were returned to normoxia. When letters above each bar are the same, there was no significant between-group difference for each nesting tactic in hatching success (Bonferroni corrected Chi-squared test with 21 pair-wise comparisons; $p \leq 0.05$). A line and asterisk (*) above treatment group indicates significant between-nesting tactic difference in hatching success within each treatment according to a Fisher's exact test ($p \leq 0.05$).

5.4.3 Embryonic mortality

There was significant between-treatment variation in the proportion of embryos that died at each stage of development (X^2_{CMH} = 143.2, d.f. = 18, p < 0.0001; Table 5.4), with no interaction with nesting strategy (Woolf test X^2 = 0.01, d.f. = 1, p = 0.92). That is, the proportion of early stage death (Stage 0) generally increased with the duration eggs spent in hypoxia (Table 5.4).

5.4.4 Hatchling morphology

Hatchling mass (g) varied significantly between treatment groups (F = 17.24, d.f. = 5, p < 0.0001), and between nesting tactics (F = 132.30, d.f. = 1, p < 0.0001), with no significant interaction (F = 1.98, d.f. = 3, p = 0.12). Hatchling mass decreased with increasing time spent in hypoxia for the arribada eggs, whilst for the non-arribada eggs hatchlings were largest in the 3-day hypoxia treatment, and arribada hatchlings were usually smaller within each treatment than non-arribada hatchlings (Table 5.5).

Hatchling head width (mm) also varied significantly between treatment groups (F = 5.19, d.f. = 5, p < 0.001), and between nesting tactics (F = 6.47, d.f. = 1, p < 0.05), with a significant interaction (F = 2.66, d.f. = 3, p = 0.05). Post-hoc analysis showed that head width varied little across treatments for the non-arribada eggs (Table 5.5). However, it differed amongst treatments for the arribada eggs, with a trend for narrower heads with increased time spent in hypoxia (Table 5.5). Non-arribada hatchlings only had larger head width within the 4-day treatment (Table 5.5).

Hatchling carapace length (mm) also varied significantly among treatment groups (F = 9.51, d.f. = 5, p < 0.0001), and between nesting tactics (F = 41.72, d.f. = 1, p < 0.0001), with no significant interaction (F = 1.26, d.f. = 3, p = 0.29). According to a Tukey's post-hoc test

carapace length decreased with increasing time spent in hypoxia for the arribada eggs, whilst for the non-arribada hatchlings carapace length did not differ significantly across treatment.

The arribada hatchlings were usually smaller within each treatment than non-arribada hatchlings (Table 5.5). Carapace width (mm) showed a similar pattern to carapace length (Table 5.5), with significant between-treatment variation ($F = 3.79$, d.f. = 5, $p < 0.01$), no significant variation between nesting tactics ($F = 2.22$, d.f. = 1, $p = 0.13$), and a significant interaction ($F = 4.05$, d.f. = 3, $p < 0.01$).

Table 5.4. Comparison between treatments of the number (%) of embryos to die at each of Leslie *et al.*'s (1996) four developmental stages.

Nesting tactic	Stage	Control	3-day	3.5-day	4-day	8-day	15-day	30-day
Non-arribada	0	7 (32%)	1 (13%)	20 (44%)	10 (30%)	28 (52%)	52 (100%)	54 (100%)
	1	2 (9%)	0 (0%)	5 (11%)	2 (6%)	5 (9%)	0 (0%)	0 (0%)
	2	2 (9%)	1 (13%)	5 (11%)	5 (15%)	6 (11%)	0 (0%)	0 (0%)
	3	11 (50%)	6 (75%)	15 (33%)	16 (48%)	15 (28%)	0 (0%)	0 (0%)
Treatment comparison*		A	A	A	A	A	A	A
Arribada	0	12 (32%)	17 (52%)	31 (78%)	24 (71%)	24 (67%)	25 (76%)	42 (100%)
	1	8 (21%)	6 (18%)	6 (15%)	2 (6%)	3 (8%)	1 (3%)	0 (0%)
	2	7 (18%)	6 (18%)	0	8 (24%)	1 (3%)	1 (3%)	0 (0%)
	3	11 (29%)	4 (12%)	3 (8%)	0 (0%)	8 (22%)	6 (18%)	0 (0%)
Treatment comparison*		A	A	AB	AB	AB	AB	B

Olive ridley eggs were collected from non-arribada and arribada nesting females and incubated following different durations of post-oviposition hypoxia.
*When letters are the same, there was no significant between-group difference in hatching success (Bonferroni corrected Chi-squared test with 21 pair-wise comparisons; $p \leq 0.05$).

Table 5.5. Mean hatchling traits for each treatment.

	Nesting tactic	Control	3-day	3.5-day	4-day	8-day	15-day	30-day
No. hatchlings measured	Non-arribada	20	20	8	20	0	0	0
	Arribada	9	11	2	6	6	8	0
Mass (g)	Non-arribada	$16.7 \pm 0.2^{A*}$	$17.8 \pm 0.3^{B*}$	16.6 ± 0.3^{AB}	$16.5 \pm 0.2^{A*}$	-	-	-
	Arribada	$14.3 \pm 0.4^{XY*}$	$14.8 \pm 0.3^{X*}$	15.0 ± 1.5^{XY}	$12.8 \pm 0.5^{Y*}$	13.7 ± 0.7^{XY}	$13.3 \pm 0.3_{XY}$	-
Head Width (mm)	Non-arribada	14.2 ± 0.1^{A}	14.4 ± 0.1^{A}	14.3 ± 0.1^{A}	$14.3 \pm 0.1^{A*}$	-	-	-
	Arribada	14.1 ± 0.1^{XY}	14.4 ± 0.1^{X}	14.3 ± 0.3^{XY}	$13.6 \pm 0.1^{Y*}$	13.8 ± 0.1^{XY}	$13.7 \pm 0.1_{Y}$	-
Carapace Length (mm)	Non-arribada	$41.3 \pm 0.3^{A*}$	$41.8 \pm 0.4^{A*}$	40.8 ± 0.4^{A}	$41.1 \pm 0.4^{A*}$	-	-	-
	Arribada	$39.1 \pm 0.4^{XY*}$	$40.1 \pm 0.4^{X*}$	40.1 ± 0.1^{XY}	$38.1 \pm 0.2^{XY*}$	39.1 ± 0.4^{XY}	$37.6 \pm 0.5_{Y}$	-
Carapace Width (mm)	Non-arribada	34.1 ± 0.16^{A}	33.9 ± 0.4^{A}	33.8 ± 0.3^{A}	$33.9 \pm 0.3^{A*}$	-	-	-
	Arribada	33.4 ± 0.4^{XY}	34.4 ± 0.4^{X}	34.8 ± 1.8^{XY}	$31.8 \pm 0.6^{Y*}$	33.1 ± 0.8^{XY}	$31.9 \pm 0.3_{Y}$	-

Olive ridley eggs were collected from non-arribada and arribada nesting females and incubated following different durations of post-oviposition hypoxia. When superscript letters are the same, there was no significant between-group difference within nesting tactic according to an ANOVA and Tukey's HSD post-hoc test. An asterisk (*) denotes a significant difference within each treatment between each nesting tactic according to an ANOVA and Tukey's HSD post-hoc test.

5.5 Discussion

Olive ridley females that nested during an arribada oviposited eggs that were capable of extending embryonic arrest for longer than eggs from those that did not nest during an arribada. Some arribada eggs still successfully hatched after 15 days in hypoxia, whereas non-arribada eggs were only capable of surviving to hatch after four days in hypoxia. To our knowledge, this is the first such finding of a developmental difference between the eggs of arribada and non-arribada nesting females. Our finding provides support for the suggestion that females nesting according to the two different behavioural tactics differ physiologically, potentially in relation to nutrition, age, and size of the turtle (Kalb 1999), because we found arribada eggs to have the greatest capability to extend arrest. Possibly less mature, or older, or physiologically compromised females are unable to produce eggs capable of maintaining embryonic arrest for as long as females that are at their physical and reproductive performance peak. Nesting in an arribada requires increased oviducal egg retention because the inter-nesting period is longer (Plotkin *et al.* 1997; Kalb 1999; Bernardo and Plotkin 2007; Rostal 2007). Furthermore, it has been reported that when the inter-nesting period between arribada events is sufficiently extended turtles that nested in the first arribada will start to change behaviours and nest solitarily before the second arribada event commences (Kalb 1999). However, we did not know the inter-nesting interval of the mothers and as such cannot rule out the unlikely possibility that the non-arribada females may have simply been late or early for an arribada. Clearly though, the ability to maintain pre-ovipositional embryonic arrest is critical for the evolution of this fascinating strategy.

Generally, the eggs from the non-arribada nesters had greater hatching success than those of arribada nesters, although this difference was only statistically significant for the control, three- and four-day hypoxia treatments. The reduced hatching success, even in the control

arribada eggs, was not due to infertility because embryos were present but it could be due to the longer period that we assume they spent in pre-ovipositional embryonic arrest in the mother's oviducts prior to the arribada and oviposition (Rostal 2007). It is generally known that greatly extended periods of arrest reduce hatching success (Kennett *et al.* 1993; Rings *et al.* 2015). Furthermore, olive ridley nests at arribada beaches generally have much lower hatching success (< 35%; Bézy *et al.* 2014) than at beaches with solitary nesters (> 75%; Dornfeld *et al.* 2015). However, there was much greater hatching success for arribada oviposited clutches in a study where they were moved to hatchery nest sites treated to reduce fungal and bacterial abundance (Bézy *et al.* 2015), suggesting that nest conditions not reproductive strategy are causing reduced hatching success. Perhaps the arribada eggs were less tolerant than non-arribada eggs to the transportation back to the laboratory, which would explain why even the control eggs had lower hatching success. Further investigation of the development of arribada and non-arribada eggs (from both arribada and solitary-only beaches) under ideal incubator conditions is warranted to ascertain differences in development.

The large proportion of eggs that formed white spots whilst in hypoxia, and the formation of a white spot on the bottom of an egg, suggests that the embryos ability to maintain arrest is compromised by prolonged hypoxia, regardless of the reproductive tactic of the mother. For the eggs that were capable of maintaining arrest, white spots then formed within one day of exposure to normoxia. Our findings also provide evidence that even if a white spot forms during hypoxic incubation, indicating the breaking of arrest, it is still possible that the egg will hatch once it is returned to normoxia, at least in this species. However, we also found that the majority of eggs in the longer hypoxic treatments formed white spots even though hatching success was reduced. Whilst white spot formation usually indicates a developing embryo, our findings provide further evidence that formation of a white spot does not always

indicate that development is successfully occurring. Indeed, the process may sometimes be affected by passive abiotic factors (Williamson *et al.* 2017).

The difference in hatchling size between treatments was unexpected. Although, the biological significance of our findings is hard to determine because there was no linear trend in hatchling size with increasing time spent in hypoxia. Potentially, extended embryonic arrest reduces the time available for development and/or reduces the capability of the embryo to assimilate egg resources. We found support for the former, because the time until first emergence of hatchlings from the nest was not delayed by a length of time equivalent to the time the eggs spent in hypoxia (i.e. embryos caught up some of the delayed development). This suggests there may be important ecological ramifications if mothers maintain eggs in arrest for longer periods through increased inter-nesting intervals. Extended embryonic arrest has been shown to impact hatchling morphology and fitness in the flatback turtle (Rings *et al.* 2015). Alternatively, the differences could be a result of mostly, or only, smaller eggs from smaller females surviving to hatching in the longer hypoxia treatments.

We also found a difference in hatchling size between arribada and non-arribada eggs, however this may be an artefact of the mother's size. It is well established that hatchling size is related to egg and female size (Bjorndal and Carr 1989; Burgess *et al.* 2006; Ischer *et al.* 2009; Booth *et al.* 2013; Sim *et al.* 2014). Therefore, the difference we found could have been an artefact of differences between females. However, we were unable to ascertain maternal identity of hatchlings in the current study, so were unable to assess between-female hatchling size variation here. Future investigation of the impact of arribada and solitary nesting behaviours on ridley hatchling morphology and fitness is warranted (Bernardo and Plotkin 2007).

In conclusion, we found differences in egg development between females that nested in arribada and non-arribada events. Arribada laid eggs had lower hatching success when incubated in normoxia or for short periods (≤ 4 days) in hypoxia, but are paradoxically capable of maintaining pre-ovipositional embryonic arrest for longer. From our data we think that pre-ovipositional arrest is an integral mechanism enabling ridley turtles to synchronise arribada nesting behaviour. Our findings provide new information on this interesting reproductive tactic and our understanding of its evolution and ecological implications. Future research is warranted to further investigate developmental processes that allow this unique reproductive tactic to occur.

5.5 Acknowledgements

S.W. received financial support throughout his PhD from an Australian Postgraduate Award. S.W.'s PhD project was supported by the Holsworth Wildlife Research Endowment – Equity Trustees Charitable Foundation. We are grateful for the assistance provided by the numerous Earthwatch Institute volunteers both in the field and laboratory. We are very thankful for the help provided by J. Black, B. Butler, L. Cruz, L. Domico, T. Dornfeld, L. Hackney, C. Hertz, L. McKenna, F. Paladino, G. Shillinger, E. Solano and J. Spotila at the Leatherback Trust and the Goldring-Gund Marine Biology Station. We appreciate the support of R. Zúñiga-Muños and the Area de Conservación Tempisque for our research. We thank P. Santidrián-Tomillo for assistance with permit applications. Thank you to T. Neeman for statistical advice. Y. Arguello-Gómez and C. Orrego helped with coordinating logistics in the field at Playa Ostional. We are very appreciative and thankful for the support and use of facilities offered by R. Piedra and the staff at Parque Nacional Marino Las Baulas. Finally, thanks to C. Neilson for invaluable assistance and support in the field and laboratory.

5.6 References

Bernardo, J, Plotkin, PT (2007) An evolutionary perspective on the arribada phenomenon and reproductive behavioral polymorphism of olive ridley sea turtles (*Lepidochelys olivacea*). In 'Biology and conservation of ridley sea turtles.' (Ed. PT Plotkin.) pp. 58-87. (The John Hopkins University Press: Baltimore, Maryland)

Bézy, VS, Valverde, RA, Plante, CJ (2014) Olive ridley sea turtle hatching success as a function of microbial abundance and the microenvironment of *In Situ* nest sand at Ostional, Costa Rica. *Journal of Marine Biology* **2014**, Article ID 351921.

Bézy, VS, Valverde, RA, Plante, CJ (2015) Olive ridley sea turtle hatching success as a function of the microbial abundance in nest sand at Ostional, Costa Rica. *PLoS One* **10**, e0118579.

Bjorndal, KA, Carr, A (1989) Variation in clutch size and egg size in the green turtle nesting population at Tortuguero, Costa Rica. *Herpetologica* **45**, 181-189.

Booth, DT, Feeney, R, Shibata, Y (2013) Nest and maternal origin can influence morphology and locomotor performance of hatchling green turtles (*Chelonia mydas*) incubated in field nests. *Marine Biology* **160**, 127-137.

Burgess, EA, Booth, DT, Lanyon, JM (2006) Swimming performance of hatchling green turtles is affected by incubation temperature. *Coral Reefs* **25**, 341-349.

Cornelius, SE, Ulloa, MA, Castro, JC, Mata del Valle, M, Robinson, DC (1991) Management of olive ridley sea turtles (*Lepidochelys olivacea*) nesting at Playas Nancite and Ostional, Costa Rica. In 'Neotropical wildlife use and conservation.' (Eds J Robinson, K Redford.) pp. 111-135. (University of Chicago Press: Chicago, IL)

Dornfeld, TC, Robinson, NJ, Tomillo, PS, Paladino, FV (2015) Ecology of solitary nesting olive ridley sea turtles at Playa Grande, Costa Rica. *Marine Biology* **162**, 123-139.

Eckrich, CE, Owens, D (1995) Solitary versus arribada nesting in the olive ridley sea turtles (*Lepidochelys olivacea*): A test of the predator-satiation hypothesis. *Herpetologica* **51**, 349-354.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Fordham, D, Georges, A, Corey, B (2006) Compensation for inundation-induced embryonic diapause in a freshwater turtle: Achieving predictability in the face of environmental stochasticity. *Functional Ecology* **20**, 670-677.

Hirth, HF (1980) Some aspects of the nesting behavior and reproductive biology of sea turtles. *American Zoologist* **20**, 507-523.

Ischer, T, Ireland, K, Booth, DT (2009) Locomotion performance of green turtle hatchlings from the Heron Island Rookery, Great Barrier Reef. *Marine Biology* **156**, 1399-1409.

Jensen, MP, Abreu-Grobois, FA, Frydenberg, J, Loeschcke, V (2006) Microsatellites provide insight into contrasting mating patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. *Molecular Ecology* **15**, 2567-2575.

Kalb, HJ (1999) Behavior and physiology of solitary and arribada nesting olive ridley sea turtles (*Lepidochelys olivacea*) during the internesting period. Texas A&M University.

Kennett, R, Georges, A, Palmerallen, M (1993) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Leslie, AJ, Penick, DN, Spotila, JR, Paladino, FV (1996) Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990-1991. *Chelonian Conservation & Biology* **2**, 159-168.

Licht, P (1982) Endocrine patterns in the reproductive cycle of turtles. *Herpetologica* **38**, 51-61.

Licht, P, Owens, DW, Cliffton, K, Penaflores, C (1982) Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, *Lepidochelys olivacea*. *General and Comparative Endocrinology* **48**, 247-253.

Miller, JD (1982) Development of marine turtles. University of New England.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Owens, DW (1980) The comparative reproductive physiology of sea turtles. *American Zoologist* **20**, 549-563.

Plotkin, PT, Byles, RA, Rostal, DC, Owens, DW (1995) Independent versus socially facilitated oceanic migrations of the olive ridley, *Lepidochelys olivacea*. *Marine Biology* **122**, 137-143.

Plotkin, PT, David Wm, O, Byles, RA, Rhonda, P (1996) Departure of male olive ridley turtles (*Lepidochelys olivacea*) from a nearshore breeding ground. *Herpetologica* **52**, 1-7.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

Pritchard, PCH (1969) Studies of the systematics and reproductive cycles of the genus *Lepidochelys*. University of Florida.

R Core Team (2016) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**, e21038.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Rostal, DC (2007) Reproductive physiology of the ridley sea turtle. In 'Biology and conservation of ridley sea turtles.' (Ed. PT Plotkin.) pp. 151-165. (Johns Hopkins University Press: Baltimore, Maryland)

Sim, EL, Booth, DT, Limpus, CJ, Guinea, ML (2014) A comparison of hatchling locomotor performance and scute pattern variation between two rookeries of the flatback turtle (*Natator depressus*). *Copeia* **2014**, 339-344.

Solomon, SE, Baird, T (1979) Aspects of the biology of *Chelonia mydas*. *Oceanography and Marine Biology: An Annual Review* **17**, 347-361.

Valverde, RA, Cornelius, SE, Mo, CL (1998) Decline of the olive ridley sea turtle (*Lepidochelys olivacea*) nesting assemblage at Nancite Beach, Santa Rosa National Park, Costa Rica. *Chelonian Conservation and Biology* **3**, 58-63.

Williamson, SA, Evans, RG, Reina, RD (2017) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Chapter 6. Ecological and evolutionary significance of a lack of capacity for extended developmental arrest in crocodilian eggs.

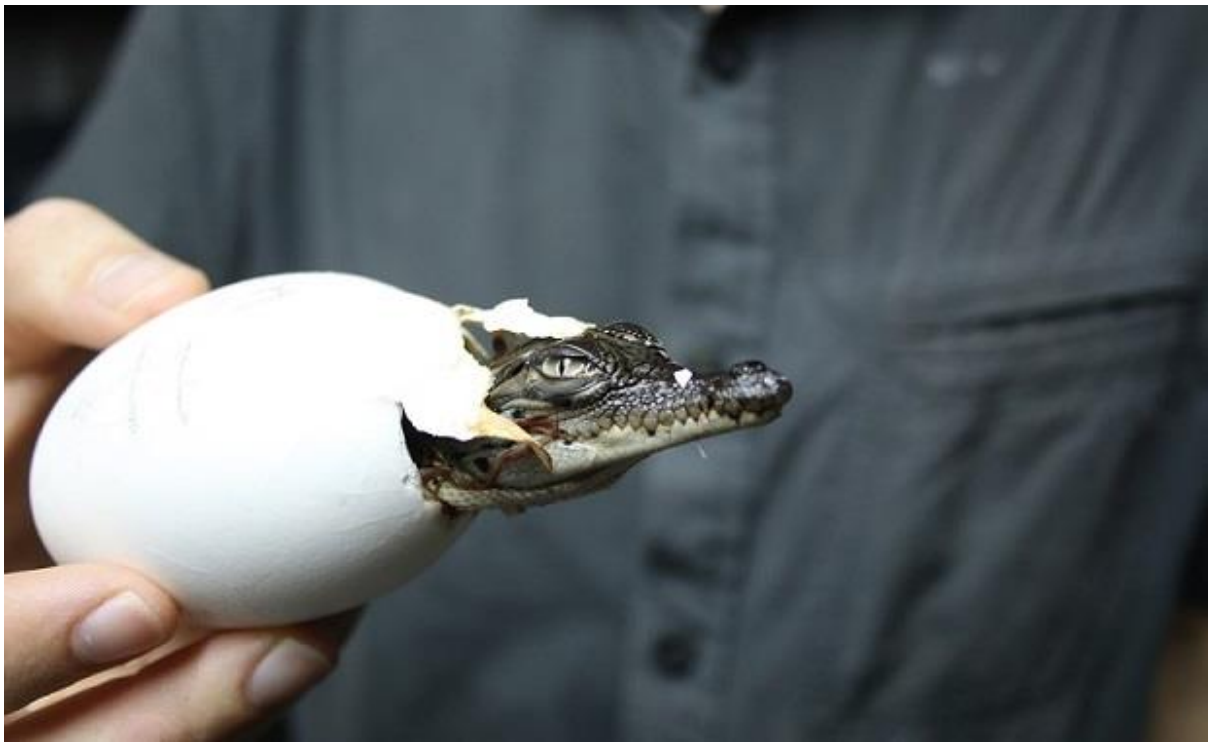
Sean A. Williamson[1], Roger G. Evans[2], S. Charlie Manolis[3], Grahame J. Webb[3], [4] and Richard D. Reina[1]

[1] School of Biological Sciences, Monash University, Clayton VIC 3800, Australia

[2] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton VIC 3800, Australia

[3] Wildlife Management International Pty Limited, [redacted] Australia

[4] Research Institute for the Environment and Livelihoods, Charles Darwin University, Darwin, NT 0909, Australia



This chapter is currently in press under the same name in *Royal Society Open Science*.

6.1 Abstract

Hypoxia within the oviducts maintains embryonic arrest in turtles at the pre-ovipositional stage, which expands the timeframe over which nesting can occur without compromising embryo survival. The arrest can be extended post-oviposition through incubation of eggs in hypoxia. We determined whether crocodilian embryos have this same capacity. We also tested whether increased oxygen availability during incubation alters hatching success. We incubated freshly-laid saltwater crocodile (*Crocodylus porosus*) eggs (N = 83) were incubated at 32°C in one of five treatments; control (normoxia; 21% O_2), 3-day and 6-day hypoxia (1% O_2), or 3-day and 6-day hyperoxia (42% O_2). Incubation (~82 days) was then completed in normoxia. There was a significant effect of treatment on survival of embryos through to hatching ($p < 0.001$). The hypoxic treatments resulted in almost no hatching (6.7% and 0% survival for the 3- and 6- day treatments respectively), while the hyperoxic and control treatments resulted in normal to high hatching success (86.6%, 100% and 64.2% for the control, 3- and 6- day hyperoxic treatments respectively). Unlike turtles, hypoxic incubation of crocodile eggs failed to delay development. Our results provide the first experimental evidence that, unlike turtles, crocodiles do not exhibit embryonic arrest when incubated under hypoxic conditions immediately following oviposition. An absence of embryonic arrest is of ecological and evolutionary significance, as it implies that crocodilians lack an ability to avoid adverse environmental conditions through delayed nesting and that, unlike turtles, pre-ovipositional arrest is not a potential explanation for the lack of viviparity in the order *Crocodylia*.

Keywords: *Embryonic Arrest, Hypoxia, Estuarine Crocodile, Nesting*

6.2 Introduction

Turtles and crocodilians exhibit a mixture of K- and r-selected species traits (Stearns 1976; Shine 2005). That is, they are all relatively long-lived species, with low adult mortality and take many years to reach maturity (K-selected traits) (Webb *et al.* 1983a; Castanet 1994; Shine and Iverson 1995; Lance 2003). However, they are oviparous (egg-laying), produce a large number of small offspring and experience high rates of mortality during the life stages before maturity (r-selected traits) (Shine 1988; Janzen *et al.* 2000; Somaweera *et al.* 2013). Crocodilians and turtles both lay eggs with relatively undeveloped embryos, having only reached the neurulation or gastrulation stages of development (Ewert 1985; Ferguson 1985; Miller 1985). Thus, they must nest on land and lay relatively undeveloped eggs, even though the majority of species are aquatic. However, some extinct archosaurs (the class of reptiles to which crocodilians belong) are thought to have been viviparous and able to sever their connection with the terrestrial environment completely (Liu *et al.* 2017). In contrast, because all crocodilians and turtles must deposit eggs into a nest, the reproductive success of the female and the phenotype of any resulting hatchlings are both heavily influenced by the abiotic and biotic conditions of the nest environment (Webb *et al.* 1987a; Packard and Packard 1988; Webb and Cooper-Preston 1989; Whitehead *et al.* 1990; Wallace *et al.* 2004; Ralph *et al.* 2005; Booth 2006).

To potentially offset exposure to adverse incubation conditions, turtles are able to improve reproductive success by delaying oviposition until abiotic and biotic conditions are more favourable (Kennett *et al.* 1993a; Kennett 1999; Rafferty and Reina 2012). They do this by arresting embryonic development *in utero* (i.e. pre-oviposition) during the gastrulation stage of development (Ewert 1985; Miller 1985). Marine turtles may be able to delay nesting by up to nine weeks (Plotkin *et al.* 1997) and some freshwater species can delay nesting for many

months (Buhlmann *et al.* 1995). However, there is evidence that extended periods of embryonic arrest through delayed nesting increases early-stage embryonic mortality in leatherback turtles, which typically have naturally low hatching success (Bell *et al.* 2003; Rafferty *et al.* 2011). Pre-ovipositional embryonic arrest is maintained by the low oxygen availability in turtle oviducts (Rafferty *et al.* 2013) and the arrest is broken by the increase in oxygen availability that occurs when eggs are laid into the nest (Kennett *et al.* 1993b; Rafferty *et al.* 2013; Rings *et al.* 2015; Williamson *et al.* 2017a). Around 12 hours of normoxia (~21% oxygen) is required to break the arrest in turtles and eggs placed into hypoxia (~1% oxygen) within 12 hours of oviposition maintain arrest and are protected from movement-induced mortality (Williamson *et al.* 2017a). When arrest breaks the embryo transitions from a gastrula into the neurulation stage of development and proceeds to completion (Ewert 1985).

In general there is limited understanding of the early developmental stages of crocodilians (Ferguson 1985), particularly regarding development of eggs whilst in the oviduct (Clarke 1891), as most developmental studies have been conducted on eggs that were at least a few hours old (Magnusson and Taylor 1980; Webb *et al.* 1983c; Ferguson 1985; Webb *et al.* 1987a). It is often difficult to obtain and study freshly laid crocodilian eggs, due to maternal protection of the nesting site. However, we know that unlike eggs of all turtle species investigated to date, crocodilian eggs are laid at the neurulation (i.e. more advanced) stage of development and have already developed 10-20 somites (Ferguson 1985), leading to the view that they may not arrest (Ferguson 1985; Ewert 1991; Rafferty and Reina 2012), although there is some disagreement (Packard *et al.* 1977; Ferguson 1985). Thus, investigation of whether crocodilians also have the ability to arrest development prior to oviposition is warranted. Extended retention of eggs by crocodilians due to adverse environmental conditions has been reported (Reese 1931; McIlhenny 1934; Ferguson 1985), supporting the

existence of pre-ovipositional arrest. However, there is anecdotal evidence from captive crocodilians that they have limited or no scope for developmental arrest during extended retention of eggs (Ferguson 1985). For example, captive females kept in high densities or without access to appropriate nesting sites will lay eggs into the water. Others have been observed to delay nesting (possibly mediated by elevated corticosteroid levels) but with reduced viability of eggs because the embryos were at a more advanced stage of development and may have already adhered to the shell membrane (Ferguson 1985). Furthermore, in contrast to turtle eggs, chilling of crocodilians eggs usually results in embryonic mortality (unpublished data reference in Ferguson 1985). These apparently contradictory observations have not been investigated experimentally.

Delayed nesting in crocodilians sometimes interferes with the formation of the opaque white spot on the shell, which normally forms on the uppermost part of the egg within 24 hours post-oviposition (Ferguson 1985; Webb *et al.* 1986; Webb *et al.* 1987b; Webb *et al.* 1987c). The white spot forms as a consequence of the embryo facilitating the movement of water from the albumin into the yolk beneath it, which at the time of laying results in rotation of the yolk bringing the embryo to the top with sub-embryonic fluid beneath it (Webb *et al.* 1987c). Continued dehydration of the albumin and the eggshell membrane creates the opaque spot, where the shell and vitelline membranes fuse, with the embryo located under that spot (Webb *et al.* 1987b; Webb *et al.* 1987c).

If development continues *in utero* such that vitelline membrane attachment occurs within the oviducts, rather than after oviposition, the yolk cannot rotate and embryos attach at random locations rather than at the top. The sub-embryonic fluid then percolates through the yolk to the top, and in the absence of that bathing fluid, embryos not attached at the top of the egg die within a few days. The presence of sub-embryonic fluid at the time of laying, which can be

detected by candling, identifies unfertilised eggs (or eggs with very early embryonic death) (Webb *et al.* 1987c). Furthermore, the location of the spot indicates whether the oviposition was delayed to the extent that embryos attached prior to egg laying. Post-oviposition, the rate at which the opaque spot forms and spreads can be altered by changing embryonic developmental rate, for example by altering incubation temperature (Webb *et al.* 1987a). Although the formation and spread of the opaque spot appear similar in turtles and crocodilians, we do not know if arrested embryonic development experienced by turtles also occurs in crocodilians or whether they have the same response to hypoxia during development.

An interesting role has been suggested for hyperoxia during development, and some crocodilian farmers incubate crocodilian eggs under hyperoxic conditions (26% O_2) for reputed benefits to hatchling fitness (Nick Stevens pers. comm.). Long-term incubation in hyperoxia (30% O_2) results in faster post-hatching growth of American alligators (Owerkowicz *et al.* 2009), but there are no reports on the impact of changes in oxygen availability during the early stages of embryonic development in crocodilians when the developmental trajectory may be determined. Whether increased oxygen availability influences the rate at which opaque white spots form remains to be determined.

Embryonic arrest has been suggested to constrain the evolution of viviparity in turtles (Rafferty *et al.* 2013; Williamson *et al.* 2017a), which along with crocodilians are among the vertebrate groups to never have evolved live-birth (Andrews and Mathies 2000). Given the important ecological and evolutionary implications that embryonic arrest may have, we aimed to determine whether crocodiles have the ability to arrest development prior to oviposition. To do this we assessed whether saltwater crocodile embryos were able to survive hypoxia by delaying development, as turtles can. We assumed that if crocodiles do not

exhibit pre-ovipositional embryonic arrest, they would be unable to delay development during hypoxia, embryos would be adversely effected, and survival to hatching would be reduced. We also assessed the impact of hyperoxia on development in order to determine whether it has any impact upon developmental success and hatchling fitness.

6.3 Material and methods

6.3.1 Egg collection

Saltwater crocodile (*Crocodylus porosus*) eggs were collected from three captive females during or within 10 min of completion of oviposition in pens at Crocodylus Park (Berrimah, Northern Territory, Australia). Eggs were quickly candled (i.e. egg contents were illuminated by a torch placed on the side of the egg) to detect sub-embryonic fluid, with any unfertilised eggs discarded.

6.3.2 Oxygen treatments

A set of treatments were used to test how changes in the partial pressure of oxygen (PO_2) affect embryonic development in saltwater crocodiles. Eggs (N = 83; total from the three clutches) were evenly distributed between five oxygen treatments (Table 6.1) after one egg from each clutch was opened at the time of egg collection to determine the approximate embryonic stage according to Ferguson's (1985) 28-stage developmental chronology (Ferguson 1985; Webb *et al.* 1987a). The remainder of the eggs (N = 80) were immediately transferred into airtight Perspex containers (Resi-Plex Plastics, North Geelong, Australia). The eggs were placed on a wire mesh allowing them to sit above approximately 10 ml of water at the base of each box.

Table 6.1. Allocation of saltwater crocodile eggs amongst experimental treatments (*N* = 83).

Treatment	Eggs	Eggs opened (day post-oviposition)	Oxygen concentration & duration of treatment
Control	21*	5 (3 at oviposition, 1 on each of days 3 and 6)	Normal atmospheric oxygen (20.9%), 6 days
Hyperoxia-3	16	1 (day 3)	~ 42% O_2, 3 days
Hyperoxia-6	15	1 (day 6)	~ 42% O_2, 6 days
Hypoxia-3	16	1 (day 3)	~ 1% O_2, 3 days
Hypoxia-6	15	1 (day 6)	~ 1% O_2, 6 days

*One additional egg was removed from the control treatment for analyses as it contained two embryos.

The experimental gases were created using 100% nitrogen for the hypoxia treatment and 42% O_2 in nitrogen for the hyperoxia treatment (Air Liquide, Australia). Each gas was humidified by bubbling it through a chamber filled with water prior to flowing the gas through each container using the inflow and outflow valves. Gas was administered for three minutes at a flow rate of eight litres per minute for each container. Ambient air was circulated through each container in the control treatment for three minutes. The PO_2 of 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). The containers were then sealed and placed in an incubator at 32.0 ± 0.2°C and 100% humidity. The maximum time between oviposition of the first egg in each clutch and the placement of all eggs in their respective treatments was approximately one hour. The Perspex boxes were re-gassed approximately every 24 hours over the treatment period (three or six days).

6.3.3 Egg development and hatching success

Eggs were checked twice daily (morning and afternoon) for the presence and position of the opaque white spot that forms on the shell. At the completion of the three- and six-day experimental treatments one egg from each treatment was opened to determine embryonic

stage. An extra egg was also opened from the control treatment at three days post-oviposition, so that embryonic stage at three and six days post-oviposition could be determined for all treatments. All remaining eggs (N = 74) were then placed into plastic trays and returned to the incubator for completion of development in normoxic conditions (21% O_2). Any eggs that were determined to have died during incubation (identified by the appearance of fungus or discolouration) were removed and opened to determine embryonic stage at death. Towards the end of the 80-day incubation period, eggs were separated by a tray divider, and checked twice daily (morning and afternoon) for any newly emerged hatchlings. Hatching success was calculated for each treatment as the proportion of hatched eggs of the total number of eggs (excluding eggs that were used for embryo staging).

6.3.4 Hatchling morphology and fitness

Hatchlings were housed individually in plastic trays in the incubator until two days post hatching, allowing absorption of excess yolk, after which the morphology and an index of fitness for each hatchling was derived. Total length (± 1 mm), snout vent length (SVL; to the front of the cloaca), head width, limb lengths, maximum belly width, and maximum yolk scar width (all ± 0.01 mm) and mass (± 0.1 g) were recorded for each hatchling. Hatchlings were allowed to warm to 32.0 ± 0.2°C for at least 5 minutes before being subjected to running and swimming tests. The running ability of each hatchling was assessed using a 3 m PVC guttering pipe lined with moist sand (15 cm wide). The swimming ability of each hatchling was assessed using a 5.1 m PVC guttering pipe (15 cm wide) filled with water (10 cm deep). Both PVC guttering pipes were kept level. Timing (to nearest second) of a hatchling commenced as soon as they began moving at one end of the pipe and concluded once it reached the other end. The presence of the researcher at the starting point of the track, gently tapping the side of the gutter at the starting end with a plastic pipe, encouraged a

unidirectional response by the hatchling towards the opposite end. Each test was repeated three times, with an interval of at least ten minutes between each test. All tests were conducted at an air and water temperature of 32 ± 0.2°C. At the completion of testing, hatchings were placed into raising pens at the farm.

6.3.5 Staging dead embryos

All dead and opened eggs were preserved for staging by injecting approximately 4 ml of 10% neutral buffered formalin into the centre of the egg and then placing the whole egg in a specimen jar filled with 10% neutral buffered formalin. Preserved eggs were later carefully dissected following the methodology described by Webb *et al.* (1983c) using a compound microscope mounted with a camera (Leica Microsystems Pty Ltd, North Ryde, Australia).

6.3.6 Data analysis

One egg from the control treatment did not hatch and was found to contain twin embryos, so it was excluded from all calculations and analyses. One hatchling failed to internalise its yolk and died shortly after hatching, and was excluded from all analyses of morphometry and fitness. All healthy eggs that were opened and staged (N = 9) were also excluded from all calculations and analyses (Table 6.1). The time each egg spent in hypoxia was subtracted from total time since oviposition to calculate aerobic latency (time taken) till white spot formation.

Homoscedasticity and normality of continuous dependent variables were assessed using the Filgner-Killen and Shapiro-Wilks tests. Between-group differences in continuous variables were assessed using analysis of variance (ANOVA) with treatment group as the independent factor and maternal identity as a random blocking factor. Post-hoc comparisons were made using Tukey's Honest Significant Difference (HSD) test. Assumptions of normality and

homoscedasticity were violated in the cases of total and aerobic latency till white spot, hatching time, hatchling mass, SVL and total length ($p < 0.05$). Consequently, these data were analysed using Kruskal Wallis and Nemenyi post-hoc tests. Between-group differences in hatching success were assessed using Cochran-Mantel-Haenszel (CMH) tests (adjusting for maternal identity) with Bonferroni corrections for pair-wise comparisons. Post-hoc analysis of hatching success was completed using Bonferroni corrected chi-squared tests with treatment group as the independent variable. Fisher's Exact Test was used to test for between-group differences in the proportion of embryos that died at either early, mid, or late stages of development. Embryos that died between Ferguson's (1985) stages 1 to 10 were classified as early, 11 to 23 as mid, and 24 to 28 as late. All analyses were conducted using R software (R Core Team 2013). All values are presented as mean ± standard error. Two-tailed $p \leq 0.05$ was considered statistically significant.

6.4 Results

6.4.1 Opaque white spot formation and embryonic development

All eggs formed an opaque white spot on the upper surface of the egg after oviposition. The eggs used were thus all considered to have been fertilised, and there was no indication that any had been subjected to delayed oviposition because no opaque white spots formed on the sides or bottom of the eggs. However, there was significant between-treatment variation in the latency (time taken) to opaque white spot formation ($H = 40.05$, d.f. = 4, $p < 0.0001$; Figures 6.1 & 6.2a). Eggs from the three-day hypoxic treatment on average took 45 hours longer to form opaque white spots than those from the control treatment (Figure 6.2a). Eggs from the six day hypoxic treatment on average took 93 hours longer than the control (Figure 6.2a). Despite these observations being consistent with arrested embryonic development, there was a large variation in latency till opaque white spot formation for both of the hypoxic

treatments, with approximately half of the eggs from each treatment forming spots prior to removal from hypoxia (7 of 15 for 3-day hypoxia and 7 of 14 for 6-day hypoxia; Figure 6.1). Once the time spent in hypoxia was accounted for ('aerobic incubation time'; defined as total time excluding time spent in hypoxia) there was no significant between-group difference in latency to opaque white spot formation (H = 8.21, d.f. = 4, p = 0.08; Figure 6.2b).

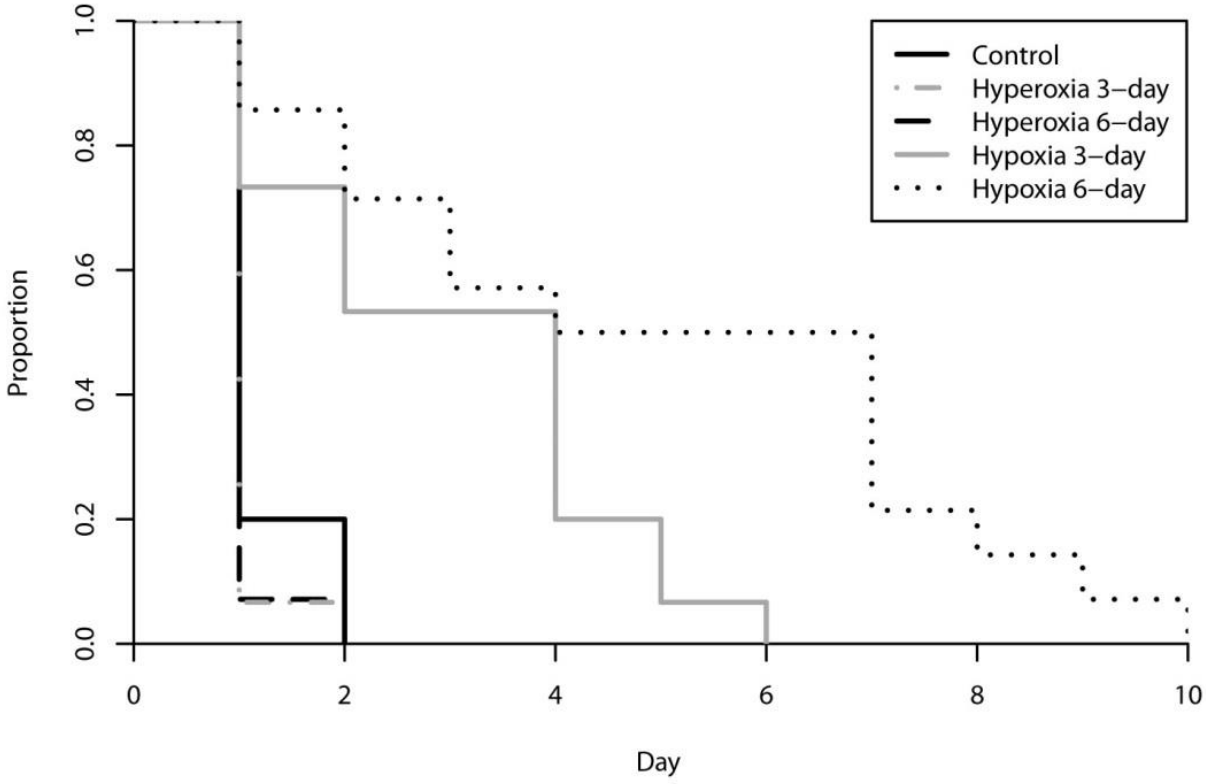


Figure 6.1. Proportion of saltwater crocodile eggs remaining to form white spots, during the first 10 days after oviposition, amongst the five treatments. Eggs (N = 73) were incubated in either normoxia (control), hyperoxia (42% O_2) for three or six days, or hypoxia (1% O_2) for three or six days (n = 14 - 15).

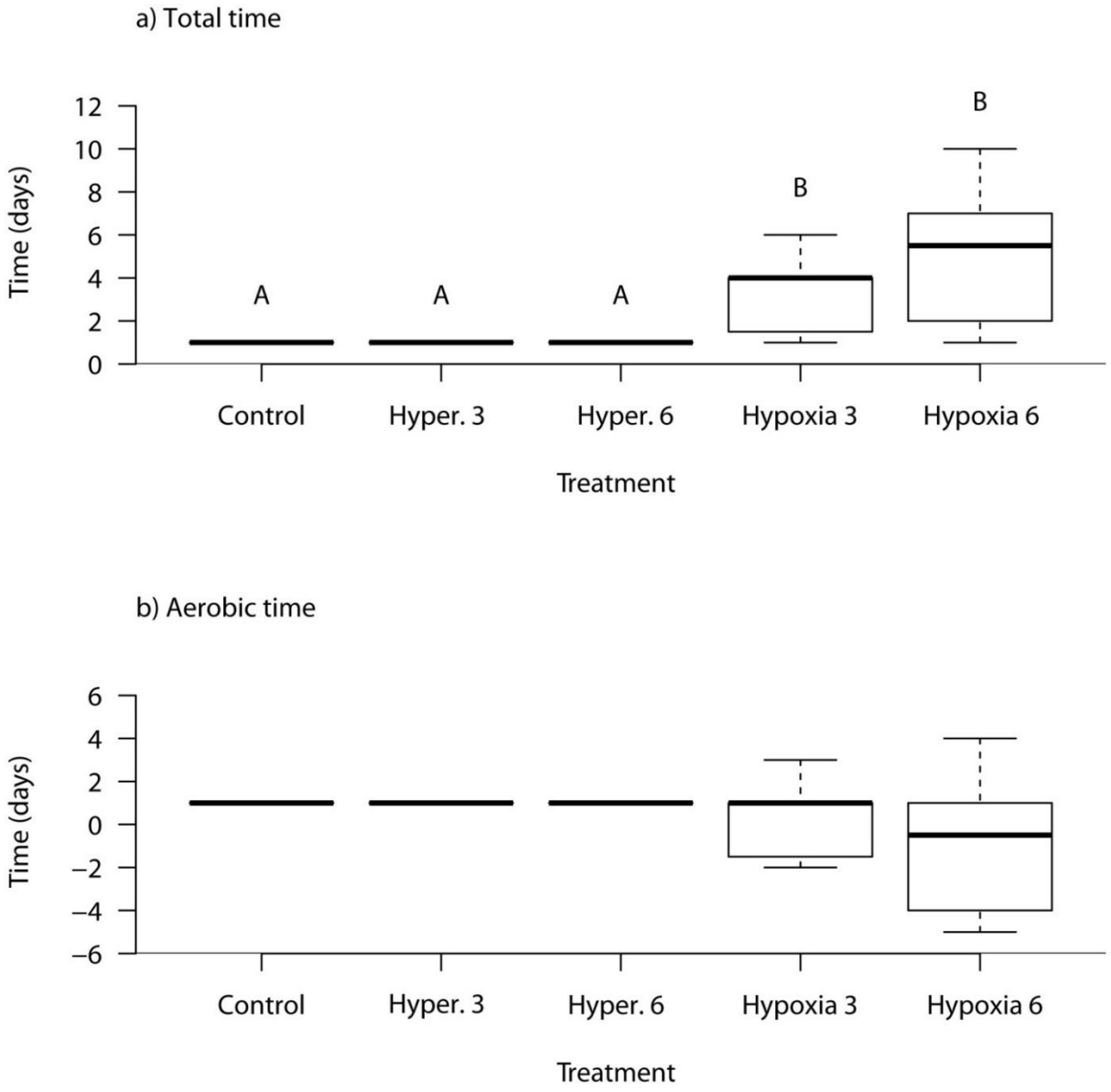


Figure 6.2. Latency from oviposition to formation of the white spot on saltwater crocodile eggs in a) total time and b) aerobic time. Eggs (N = 73) were incubated in either normoxia (control), hyperoxia (42% O_2) for three (Hyper. 3) or six (Hyper. 6) days, or hypoxia (1% O_2) for three (Hyp. 3) or six days (Hyp. 6) (n = 14 - 15). Aerobic time is the total time from oviposition excluding time spent in hypoxia. Boxplot centre lines show medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. For a) if 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; $p < 0.0001$). For b) there was no significant between-group difference (Kruskal-Wallis and Nemenyi's post-hoc test; $p > 0.05$).

The embryonic stage of eggs that were opened differed between the hypoxic and the aerobic (i.e. control and hyperoxic) treatments (Table 6.2). That is, all embryos were at the stage of development expected for their age (Ferguson 1985), except the two that were opened from the hypoxic treatments, which were either one stage behind developmental schedule (Ferguson 1985) or had not developed at all (Table 6.2).

Table 6.2. Stage of development of saltwater crocodile eggs randomly selected from the various treatments.

Day	Control	Hyperoxic treatments	Hypoxic treatments
0	1, 1, 1	-	-
3	3	3	2
6	6	6	1

Embryos (N = 9) were staged according to Ferguson's (1985) 28-stage developmental chronology. Eggs were incubated in either normoxia (control), hyperoxia (42% O_2) for three or six days, or hypoxia (1% O_2) for three or six days.

In summary, white spot formation was delayed in a non-systematic way by hypoxic incubation, whilst hyperoxic incubation did not have any detectable impact upon timing of white spot formation. Furthermore, eggs that we opened after removal from hypoxia had embryos that were behind in their predicted developmental schedule.

6.4.2 Hatching, embryonic death and hatchling traits

Hatching success varied significantly among the various treatments (X^2_{CMH} = 48.29, d.f. = 4, $p < 0.0001$; Figure 6.3) with no three-way association with female identity (Woolf test X^2 = 0.67, d.f. = 2, p = 0.71). The three aerobic treatments (control and two hyperoxic treatments) had greater hatching success (64.3% to 100%) than the two hypoxic treatments (0% to 6.7%; Figure 6.3). There was no significant between-group variation in the time taken to hatch (H = 1.10, d.f. = 3, p = 0.78). All eggs took an average of 81.5 days to hatch (Table 6.3). For eggs that failed to hatch, there was significant between-treatment variation in the proportion of embryos that died at each developmental period (Fisher's Exact Test; $p < 0.001$). Dead

embryos from treatments that had low hatching success (both hypoxia treatments) typically died early during development, whereas those from treatments that had normal hatching success for crocodiles (control and both hyperoxia treatments) died at stages throughout development (Figure 6.4). From the eggs that hatched, there was no significant between-group variation in hatchling morphology or fitness traits (Table 6.3; $p > 0.05$).

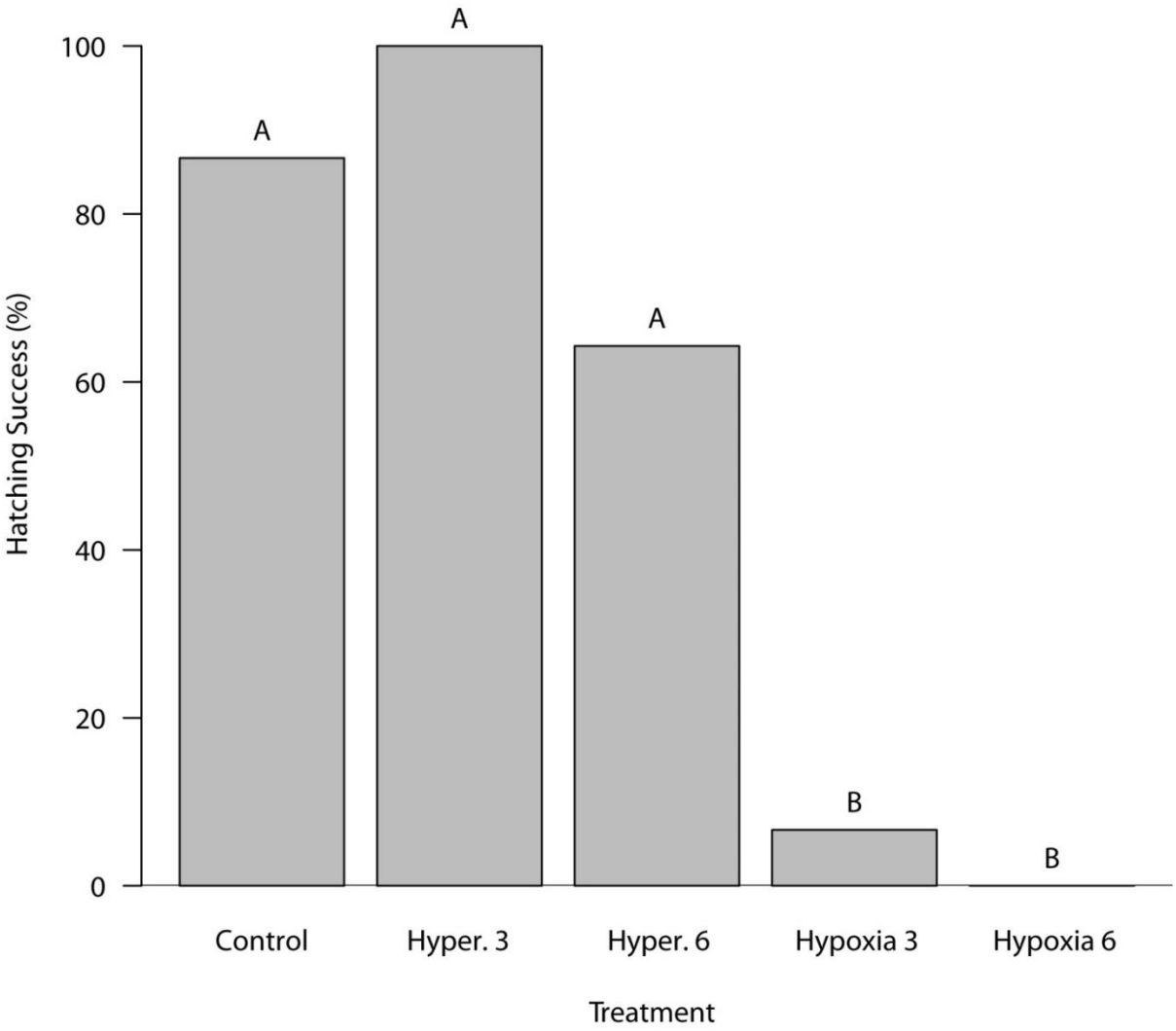


Figure 6.3. Proportion of saltwater crocodile eggs to hatch (%) after the various treatments. Eggs (N = 73) were incubated in either normoxia (control), hyperoxia (42% O_2) for three (Hyper. 3) or six (Hyper. 6) days, or hypoxia (1% O_2) for three (Hypoxia 3) or six days (Hypoxia 6) (n = 14 - 15). Following their respective treatments all eggs were incubated in normoxia until hatching. When letters above each bar are the same, there was no significant between-group difference in hatching success (Bonferroni corrected Chi-squared test with ten pair-wise comparisons; $p \leq 0.05$).

Table 6.3. Traits of saltwater crocodile hatchlings from various treatments.

	Control	Hyperoxia (3-days)	Hyperoxia (6-days)	Hypoxia (3-days)	Hypoxia (6-days)	Test statistic	*P* value
No. of hatchlings	13	15*	9	1	0	$X^2_{CMH} = 42.65$	<0.0001
Hatching time (d)	81.5 ± 0.2	81.3 ± 0.2	81.8 ± 0.5	82.0	N/A	$H_{(3,33)} = 0.78$	0.78
Mass (g)	77.0 ± 1.1	78.1 ± 0.8	76.4 ± 0.5	80.2	N/A	$H_{(3,33)} = 4.40$	0.22
SVL (mm)	143.4 ± 1.1	143.9 ± 0.7	140.1 ± 1.6	140.9	N/A	$H_{(3,33)} = 4.55$	0.21
Total Length (mm)	306 ± 4	304 ± 1	300 ± 2	299	N/A	$H_{(3,33)} = 3.71$	0.29
Head Width (mm)	23.2 ± 0.1	23.4 ± 0.5	23.0 ± 0.1	24.0	N/A	$F_{(3,27)} = 1.38$	0.37
FRLL (mm)	50.3 ± 0.4	50.2 ± 0.6	50.2 ± 0.4	50.2	N/A	$F_{(3,27)} = 0.39$	0.77
FLLL (mm)	50.7 ± 0.6	51.3 ± 0.6	50.1 ± 0.5	50.0	N/A	$F_{(3,27)} = 2.41$	0.21
BRLL (mm)	63.1 ± 0.4	63.8 ± 0.5	63.9 ± 0.6	60.8	N/A	$F_{(3,27)} = 0.85$	0.54
BLLL (mm)	62.8 ± 0.4	63.2 ± 0.6	63.0 ± 0.5	62.5	N/A	$F_{(3,27)} = 0.18$	0.90
Belly Width (mm)	39.0 ± 0.5	39.9 ± 0.4	40.8 ± 0.5	40.4	N/A	$F_{(3,27)} = 1.39$	0.37
Yolk Scar Width (mm)	4.3 ± 0.3	4.1 ± 0.4	4.9 ± 0.3	4.4	N/A	$F_{(3,27)} = 0.97$	0.49
Swim speed (cm/s)	23.0 ± 0.4	22.9 ± 0.3	23.0 ± 0.4	24.9	N/A	$F_{(3,27)} = 0.36$	0.79
Run speed (cm/s)	27.8 ± 1.0	28.7 ± 1.0	26.4 ± 1.5	28.4	N/A	$F_{(3,27)} = 3.89$	0.11

*****One hatchling failed to internalise its yolk after hatching and died. Subsequently, it was removed from analysis except for hatching time. Eggs (N = 38) were incubated in either normoxia (control), hyperoxia (42% O_2) for three or six days, or hypoxia (1% O_2) for three or six days. Following their respective treatments all eggs were incubated in normoxia until hatching. Front (F) and Back (B), Left (L) and Right (R) Leg Lengths (LL) were measured.

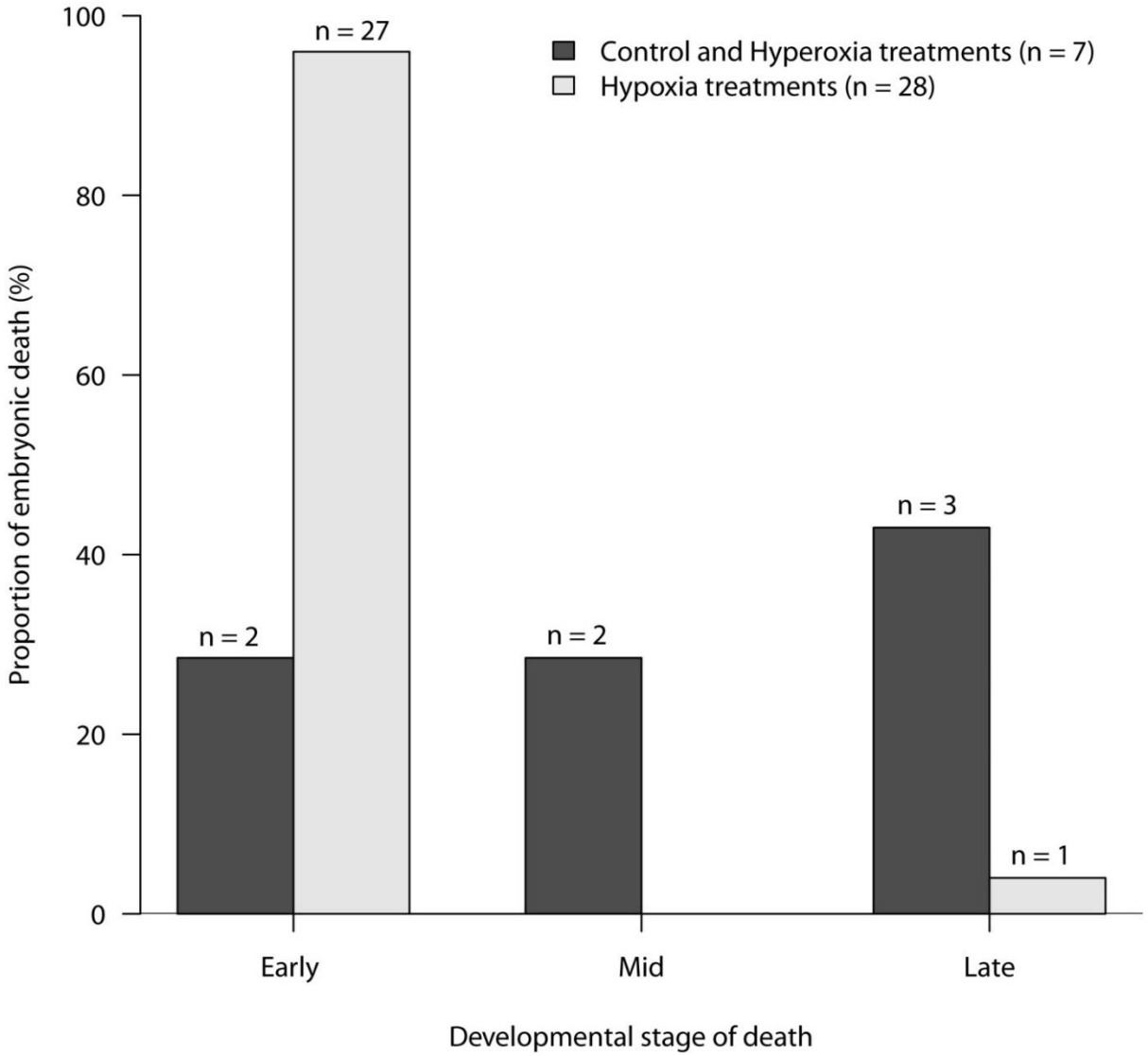


Figure 6.4. Proportion of embryonic death at three stages of development in saltwater crocodile eggs. Eggs (*N* = 73) were incubated in either normoxia (control), hyperoxia (42% O_2) for three or six days, or hypoxia for three or six days (*n* = 14 - 15). Following their respective treatments all eggs were incubated in normoxia until hatching. For this analysis, experimental groups were combined into those in which there was a low incidence of embryonic death (0 – 35.7% for control and hyperoxia treatments; 7 embryos total) and those in which there was a high incidence of embryonic death (93 – 100% for both hypoxia treatments; 28 embryos in total). Embryos were staged according to Ferguson's (1985) 28-stage developmental chronology and further classified as either early (Stages 1- 10), mid (Stages 11 – 23), or late (Stages 24 – 28).

In summary, hypoxic incubation resulted in mortality of almost all embryos. Furthermore, the embryos from the hypoxic treatments died at early developmental stages. However, between the hyperoxic and control treatments we found no difference in hatching success or stage of development for dead embryos.

6.5 Discussion

We showed that crocodile eggs cannot survive hypoxia in the way that turtle eggs can, suggesting that, unlike turtles, crocodilians may not arrest embryonic development prior to oviposition. We found that hypoxia did not completely delay development, because opaque white spots still formed and some embryos continued to grow while in hypoxia. This result is in contrast to the response shown in turtles, which exhibit pre-ovipositional embryonic arrest (Kennett *et al.* 1993b; Fordham *et al.* 2006; Fordham *et al.* 2007; Rafferty *et al.* 2013; Rafferty and Reina 2014; Rings *et al.* 2015; Williamson *et al.* 2017a). Our results appear to explain why crocodilians that are faced with adverse nesting conditions either oviposit impaired embryos or lay eggs underwater (Ferguson 1985). The ecological implication is that crocodilians have limited capacity to improve reproductive success by delaying nesting during a breeding period of sub-optimal environmental conditions if they have already commenced ovulation. However, there is anecdotal evidence that many females will oviposit their eggs during storm activity on the same day, which suggests that they can hold onto eggs for a period of time, but cannot do for long periods like turtles can.

Crocodilian eggs require sufficient oxygen immediately after oviposition for development to continue successfully to hatching. Our results show that an extremely hypoxic incubation environment of 1% oxygen (PO_2 ~8 mmHg) for three to six days, commencing within 1 h of oviposition, led to embryonic death at early stages of development and so significantly reduced hatching success. This contrasts with what has been found for turtle embryos, which arrest development in hypoxia and usually recommence development once returned to normoxia (Kennett *et al.* 1993b; Kennett *et al.* 1998; Fordham *et al.* 2007; Williamson *et al.* 2017a), although some effects on subsequent hatching success have been observed (Kennett *et al.* 1993b; Rafferty *et al.* 2013; Rings *et al.* 2015). Flooding of nesting habitat inundates

crocodilian nests and is a common cause of mortality (Webb *et al.* 1977; Magnusson 1982; Webb *et al.* 1983b; Kushlan and Mazzotti 1989; Elsey and Trosclair III 2008; Cedillo-Leal *et al.* 2017). If this were to occur for three days at the start of development of a clutch of eggs, our findings suggest that the hypoxic conditions (<1% O_2 availability in water) created would be lethal for the embryo. Indeed, there was total mortality of alligator eggs that were subjected to experimental flooding for two days (Joanen *et al.* 1977).

We found no detectable impact of hyperoxic incubation on crocodilian development or hatchlings. Hyperoxia for three or six days after oviposition did not change developmental timing, hatching success or hatchling fitness and morphology. Our findings are consistent with the only other similar experiment reported, in which there was also minimal impact of short-term hyperoxia during early incubation of flatback sea turtle eggs (*Natator depressus*; Rings *et al.* 2015). However, it has been shown that smaller increases in oxygen availability than we used (30% vs 42% O_2), but during later stages of development, have positive impacts upon embryonic development and hatchling fitness of American alligators (Owerkowicz *et al.* 2009). Atmospheric oxygen availability has fluctuated greatly throughout crocodilian evolutionary history (from about 13% to 31% O_2) and incubation of alligator eggs in levels of hyperoxia that were experienced have been shown to positively affect development and bone composition, with an optimum at 27% O_2 (Berner *et al.* 2007; VandenBrooks 2007). No effect of hyperoxic incubation during early development has now been shown (this study, (Rings *et al.* 2015)) for two oviparous reptiles (*C. porosus* and *N. depresssus*), but early-stage embryonic death is uncommon for both of these species (Webb and Cooper-Preston 1989; Hewavisenthi *et al.* 2001; Hewavisenthi and Parmenter 2002). However, extended pre-ovipositional embryonic arrest has been implicated in higher proportions of early-stage embryonic death in leatherback turtles (Rafferty *et al.* 2011). Further, we know that arrest is broken by an increase in oxygen availability (Kennett *et al.* 1993b; Rafferty *et al.* 2013). In a

species with relatively low hatching success, such as the leatherback turtle, it is possible that hyperoxia might stimulate development and reduce the high level of early-stage embryonic death typically found (Bell *et al.* 2003). Therefore, it seems useful to now assess how hyperoxia during early-development might affect a species with pre-ovipositional embryonic arrest and high amounts of early-stage embryonic mortality, such as the leatherback turtle (Bell *et al.* 2003).

Our findings suggest that a hypoxic environment would not be suitable for safe transportation of crocodilian eggs, because hypoxia did not delay development. Failure of hypoxia to delay development means that embryos would commence development even during transportation and would render eggs susceptible to movement-induced mortality. This contrasts with our results with turtles, where hypoxic incubation delayed development and subsequently protected eggs from movement-induced mortality (Williamson *et al.* 2017a; Williamson *et al.* 2017b). Sudden jolting of crocodile eggs during transport, between 8-12 days post-oviposition, is likely to result in embryonic mortality because the recently-formed chorioallantois is fragile. However, data from another study indicate that rotation of a crocodile egg will kill an embryo after it has attached to the shell (~1 day post-oviposition) but prior to adequate development of the respiratory and excretory functions of the allantois (Webb *et al.* 1987b). Deeming and Ferguson (1991) showed that eggs can withstand 60 degree rotations, but care should always be taken to prevent unnecessary movement of eggs (Ferguson 1982, 1985). Therefore, it is prudent that crocodilian researchers and conservationists continue to exercise caution during transport of eggs.

In order to further develop our understanding of the evolution of viviparity it is useful and interesting to understand why it has not evolved in some particular taxa (Andrews and Mathies 2000). We showed that crocodile embryos are not arrested by hypoxia at oviposition.

This lack of hypoxia-mediated embryonic arrest would be one fewer physiological constraint on the evolution of viviparity or even facultative oviparity (Webb and Cooper-Preston 1989; Williamson *et al.* 2017a). Our finding that eggs fail to develop whilst incubated in hypoxia, for even as short as three days after oviposition, suggests that increased embryonic development *in utero* would require sufficient oxygen availability to be present within the oviducts of crocodilians (Andrews 2002).

Crocodilian embryos could still be susceptible to movement-induced mortality if eggs are retained in the oviduct beyond the normal time for oviposition. Therefore obligate oviparity (eggs oviposited with an early stage embryo) may be an evolutionary one-way path that precludes subsequent evolution of advanced development *in utero* and ultimately of viviparity in this taxon (Webb and Cooper-Preston 1989). The evolution of some parental care, in the form of nest- and crèche-guarding, may have been important in the evolution of crocodilian embryonic developmental patterns (Tinkle and Gibbons 1977; Ferguson 1985; Webb and Cooper-Preston 1989). Crocodilians may experience less selection pressure to delay nesting because, unlike turtles, they are well-equipped to protect themselves and their nests from most predation.

It has also been suggested that a morphological difference in oviducts between crocodilians and other vertebrates is important for the evolution of their respective developmental patterns (Palmer and Guillette Jr 1992). Crocodilians have an "assembly-line" oviducal morphology where each region performs one task such as calcium secretion or eggshell membrane formation whilst other taxon, such as turtles, are able to perform both functions in the same region (Palmer and Guillette Jr 1992). Perhaps this difference may explain why crocodilians do not arrest development, but further investigation of the evolution of reproduction in crocodilians is needed to understand why this almost entirely aquatic taxon has remained

oviparous. It is possible that crocodilian oviducal oxygen availability is a constraint on further *in utero* development, as it may be for turtles, and warrants investigation (Rafferty *et al.* 2013).

A possible limitation in our current study results from the maximum of one hour delay between oviposition and placement of eggs into their respective treatments. Our conclusion that pre-ovipositional developmental arrest does not occur in saltwater crocodiles is based on the assumption that this very brief period of normoxia did not cause the embryos to recommence development if they were in a state of arrest when eggs were laid. However, our assumption is supported by data from green turtles showing that the eggs require at least 12 hours of normoxia after oviposition in order for pre-ovipositional embryonic arrest to be broken (Williamson *et al.* 2017a). The short delay between oviposition and placement of eggs into their respective experimental treatments is difficult to reduce or remove with crocodilians, because of safety concerns for researchers whilst trying to collect freshly-laid eggs from such an aggressive and dangerous animal. Avoiding the elapsed time between laying of the first and last eggs (10 – 45 minutes, typically), by collecting each egg as it is oviposited, cannot be achieved without considerable risk. Inducing females to lay eggs whilst restrained is not advisable because it is impossible to determine exactly when the eggs would have been laid if the female was left to nest naturally. Thus, while we think that the brief period of normoxia is unlikely to have influenced the developmental progression of the embryos, we are unable to discount this possibility.

In conclusion, we have provided experimental evidence leading us to conclude that crocodilian embryos do not undergo pre-ovipositional arrest. Our observations and conclusion are consistent with the limited anecdotal evidence from farmers and previous researchers (Ferguson 1985). Importantly, it means that unlike turtles, crocodilians have

limited capacity to avoid adverse nesting conditions by delaying nesting. The discovery of a presumptive lack of pre-ovipositional embryonic arrest in crocodilians prompts further investigation as to why this predominately aquatic taxon is dependent on obligate oviparity, especially when other aquatic archosaurs were likely viviparous. We found no detectable impact of hyperoxic incubation on crocodilian development and hatchling fitness. We suggest that hyperoxic incubation could be used to improve hatching success in species that arrest development prior to oviposition and experience high levels of early embryonic mortality. The evolutionary implications of the reproductive strategy of crocodilians suggested by our results may be an interesting avenue for investigation in understanding the prerequisites for viviparity.

6.6 Ethics statement

All experimental procedures were approved by Monash University's School of Biological Science Animal Ethics Committee (Approval BSCI/2013/24).

6.7 Data accessibility

Our data are deposited at Dryad: http://dx.doi.org/10.5061/dryad.nq41m

6.8 Competing interests

The authors declare no competing interests.

6.9 Author's contributions

SW designed and carried out the experiment, completed the data analysis and drafted the manuscript. CM and GW assisted with experimental work and helped draft the manuscript. RE and RR supervised SW and assisted with drafting the manuscript. All authors gave final approval for publication.

6.10 Acknowledgements

We would like to express our gratitude to C. Rings and A. Rafferty for help and encouragement with this project. Thanks to E. Hayward at Charles Darwin University for use of the microscope in Darwin. We thank I. Balazs for assistance at Crocodylus Park and for photographs. M. Brien offered invaluable help with logistics at Crocodylus Park, especially regarding the camera monitoring of nests. Thanks to R. Elsey for helpful discussions regarding crocodilian reproductive biology. Sincere thanks to B. Beverley and C. Neilson for volunteering their time to assist with egg collection and monitoring at Crocodylus Park. Finally, thank you to S. Ferguson, O. Plume and all staff at Crocodylus Park for assistance with this study.

6.11 Funding

B. Holsworth and the Holsworth Wildlife Research Endowment for financial support awarded to S. Williamson. We thank the IUCN-SSC Crocodile Specialist Group for the award of a Student Research Assistance Scheme grant to S. Williamson. Financial support to S. Williamson was made available by the Australian Research Council through a Research Training Program PhD Scholarship.

6.7 References

Andrews, RM (2002) Low oxygen: A constraint on the evolution of viviparity in reptiles. *Physiological and Biochemical Zoology* **75**, 145-154.

Andrews, RM, Mathies, T (2000) Natural history of reptilian development: Constraints on the evolution of viviparity. *BioScience* **50**, 227-238.

Bell, BA, Spotila, JR, Paladino, FV, Reina, RD (2003) Low reproductive success of leatherback turtles, *Dermochelys coriacea*, is due to high embryonic mortality. *Biological Conservation* **115**, 131-138.

Berner, RA, VandenBrooks, JM, Ward, PD (2007) Oxygen and evolution. *Science* **316**, 557-558.

Booth, DT (2006) Influence of incubation temperature on hatchling phenotype in reptiles. *Physiological and Biochemical Zoology* **79**, 274-281.

Buhlmann, K, Lynch, T, Whitfield Gibbons, J, Greene, J (1995) Prolonged egg retention in the turtle *Deirochelys reticularia* in South Carolina. *Herpetologica* **51**, 457-462.

Castanet, J (1994) Age estimation and longevity in reptiles. *Gerontology* **40**, 174-192.

Cedillo-Leal, C, Simoncini, MS, Leiva, PML, Larriera, A, Lang, JW, Piña, CI (2017) Eggshell structure in *Caiman latirostris* eggs improves embryo survival during nest inundation. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20162675.

Clarke, SF (1891) The habits and embryology of the American alligator. *Journal of Morphology* **5**, 181-214.

Deeming, DC, Ferguson, MWJ (1991) Egg turning during incubation has no effect upon the growth of embryos of *Alligator mississippiensis*. *Acta Zoologica* **72**, 125-128.

Elsey, RM, Trosclair III, PL (2008) Effect of timing of egg collection on growth in hatchling and juvenile American alligators. *Herpetological Bulletin* **105**, 13-18.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Ewert, MA (1991) Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In 'Egg incubation: ifs effects on embryonic development in birds and reptiles.' (Eds DC Deeming, MWJ Ferguson.) pp. 173-191. (Cambridge University Press: Cambridge, UK)

Ferguson, MWJ (1982) The structure and composition of the eggshell and embryonic membranes of *Alligator mississippiensis*. *The Transactions of the Zoological Society of London* **36**, 99-152.

Ferguson, MWJ (1985) Reproductive biology and embryology of the crocodilians. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 329-491. (John Wiley & Sons: New York)

Fordham, D, Georges, A, Corey, B (2006) Compensation for inundation-induced embryonic diapause in a freshwater turtle: Achieving predictability in the face of environmental stochasticity. *Functional Ecology* **20**, 670-677.

Fordham, DA, Georges, A, Corey, B (2007) Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise. *Aquaculture* **270**, 105-114.

Hewavisenthi, S, Parmenter, CJ (2002) Incubation environment and nest success of the flatback turtle (*Natator depressus*) from a natural nesting beach. *Copeia* **2002**, 302-312.

Hewavisenthi, S, Parmenter, CJ, Gatten Jr, RE (2001) Influence of incubation environment on the development of the flatback turtle (*Natator depressus*). *Copeia* **2001**, 668-682.

Janzen, FJ, Tucker, JK, Paukstis, GL (2000) Experimental analysis of an early life-history stage: Selection on size of hatchling turtles. *Ecology* **81**, 2290-2304.

Joanen, T, McNease, L, Perry, G (1977) Effects of simulated flooding on alligator eggs. *Proc. 31st Ann. Conf. Southeast. Assoc. Game Fish Coom.* **31**, 33-35.

Kennett, R (1999) Reproduction of two species of freshwater turtle, *Chelodina rugosa* and *Elseya dentata*, from the wet–dry tropics of northern Australia. *Journal of Zoology* **247**, 457-473.

Kennett, R, Christian, K, Bedford, G (1998) Underwater nesting by the Australian freshwater turtle *Chelodina rugosa*: effect of prolonged immersion and eggshell thickness on incubation period, egg survivorship, and hatchling size. *Canadian Journal of Zoology* **76**, 1019-1023.

Kennett, R, Christian, KA, Pritchard, D (1993a) Underwater nesting by the tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*). *Australian Journal of Zoology* **41**, 47-52.

Kennett, R, Georges, A, Palmerallen, M (1993b) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Kushlan, JA, Mazzotti, FJ (1989) Population biology of the American crocodile. *Journal of Herpetology* **23**, 7-21.

Lance, VA (2003) Alligator physiology and life history: The importance of temperature. *Experimental Gerontology* **38**, 801-805.

Liu, J, Organ, CL, Benton, MJ, Brandley, MC, Aitchison, JC (2017) Live birth in an archosauromorph reptile. *Nature Communications* **8**, 14445.

Magnusson, W, Taylor, J (1980) A description of developmental stages in *Crocodylus porosus*, for use in aging eggs in the field. *Wildlife Research* **7**, 479-485.

Magnusson, WE (1982) Mortality of eggs of the crocodile *Crocodylus porosus* in northern Australia. *Journal of Herpetology* **16**, 121-130.

McIlhenny, EA (1934) Notes on incubation and growth of alligators. *Copeia* **1934**, 80-88.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Owerkowicz, T, Elsey, RM, Hicks, JW (2009) Atmospheric oxygen level affects growth trajectory, cardiopulmonary allometry and metabolic rate in the American alligator (*Alligator mississippiensis*). *Journal of Experimental Biology* **212**, 1237-1247.

Packard, GC, Packard, MJ (1988) The physiological ecology of reptilian eggs and embryos. In 'Biology of the Reptilia.' (Eds C Gans, RB Huey.) Vol. 16 pp. 523-605. (John Wiley & Sons Inc: New York)

Packard, GC, Tracy, CR, Roth, JJ (1977) The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the class *Reptilia*. *Biological Reviews* **52**, 71-105.

Palmer, BD, Guillette Jr, LJ (1992) Alligators provide evidence for the evolution of an archosaurian mode of oviparity. *Biology of Reproduction* **46**, 39-47.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

R Core Team (2013) R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna, Austria

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Reina, RD (2014) The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. *Journal of Experimental Marine Biology and Ecology* **450**, 91-97.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**, e21038.

Ralph, CR, Reina, RD, Wallace, BP, Sotherland, PR, Spotila, JR, Paladino, FV (2005) Effect of egg location and respiratory gas concentrations on developmental success in nests of the leatherback turtle, *Dermochelys coriacea*. *Australian Journal of Zoology* **53**, 289-294.

Reese, AM (1931) The ways of the alligator. *The Scientific Monthly* **33**, 321-335.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Shine, R (1988) Parental care in reptiles. In 'Biology of the Reptilia.' (Eds C Gans, RB Huey.) Vol. 16 pp. 275-329. (John Wiley and Sons: New York)

Shine, R (2005) Life-history evolution in reptiles. *Annual Review of Ecology, Evolution, and Systematics* **36**, 23-46.

Shine, R, Iverson, JB (1995) Patterns of survival, growth and maturation in turtles. *Oikos* **72**, 343-348.

Somaweera, R, Brien, M, Shine, R (2013) The role of predation in shaping crocodilian natural history. *Herpetological Monographs* **27**, 23-51.

Stearns, SC (1976) Life-history tactics: a review of the ideas. *Quarterly Review of Biology* **51**, 3-47.

Tinkle, DW, Gibbons, JW (1977) The distribution and evolution of viviparity in Reptiles. *Misc. Publ. Mus. zool. Univ. Mich.* **154**, 1-55.

VandenBrooks, JM (2007) The effects of varying partial pressure of oxygen on vertebrate development and evolution. Yale University.

Wallace, Bryan P, Sotherland, Paul R, Spotila, James R, Reina, Richard D, Franks, Bryan F, Paladino, Frank V (2004) Biotic and abiotic factors affect the nest environment of embryonic leatherback turtles, *Dermochelys coriacea*. *Physiological and Biochemical Zoology* **77**, 423-432.

Webb, GJW, Beal, MA, Manolis, CS, Dempsey, KE (1987a) The effects of incubation temperature on sex determination and embryonic development rate in *Crocodylus johnstoni* and *C. porosus*. In 'Wildlife Management; Crocodiles and Alligators.' (Eds GJW Webb, CS Manolis, PJ Whitehead.) pp. 507-531. (Surrey Beatty: Chipping Norton, Australia)

Webb, GJW, Buckworth, R, Manolis, SC (1983a) *Crocodylus johnstoni* in the Mckinlay River Area, N.T. III. Growth, movement and the population age structure. *Wildlife Research* **10**, 383-401.

Webb, GJW, Choquenot, D, Whitehead, PJ (1986) Nests, eggs, and embryonic development of *Carettochelys insculpta* (*Chelonia: Carettochelidae*) from Northern Australia. *Journal of Zoology, Series B* **1**, 521-550.

Webb, GJW, Cooper-Preston, H (1989) Effects of incubation temperature on crocodiles and the evolution of reptilian oviparity. *Integrative and Comparative Biology* **29**, 953-971.

Webb, GJW, Manolis, CS, Dempsey, KE, Whitehead, PJ (1987b) Crocodilian eggs: A function overview. In 'Wildlife Management; Crocodiles and Alligators.' (Eds GJW Webb, CS Manolis, PJ Whitehead.) pp. 417-422. (Surrey Beatty: Chipping Norton, Australia)

Webb, GJW, Manolis, SC, Buckworth, R, Sack, GC (1983b) An examination of *Crocodylus porosus* nests in two northern Australian freshwater swamps, with an analysis of embryo mortality. *Wildlife Research* **10**, 571-605.

Webb, GJW, Manolis, SC, Buckworth, R, Sack, GC (1983c) An interim method for estimating the age of *Crocodylus porosus*. *Wildlife Research* **10**, 563-570.

Webb, GJW, Manolis, SC, Whitehead, PJ, Dempsey, K (1987c) The possible relationship between embryo orientation opaque banding and the dehydration of albumen in crocodile eggs. *Copeia* **1987**, 252-257.

Webb, GJW, Messel, H, Magnusson, W (1977) The nesting of *Crocodylus porosus* in Arnhem Land, Northern Australia. *Copeia* **1977**, 238-249.

Whitehead, PJ, Webb, GJW, Seymour, RS (1990) Effect of incubation temperature on development of *Crocodylus johnstoni* embryos. *Physiological Zoology* **63**, 949-964.

Williamson, SA, Evans, RG, Reina, RD (2017a) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Williamson, SA, Evans, RG, Robinson, NJ, Reina, RD (2017b) Hypoxia as a novel method for preventing movement-induced mortality during translocation of turtle eggs. *Biological Conservation* **216**, 86-92.

Chapter 7. General Discussion.

7.1 Summary of major findings

The over-arching aim of the studies described in this thesis was to understand the intricacies of how pre-ovipositional embryonic arrest functions and what management and conservation implications it may have. Through this work, I provided novel data on how pre-ovipositional embryonic arrest functions within turtles. I also produced data indicating that crocodilian embryos do not arrest development in hypoxia. Furthermore, I demonstrated the conservation and management implications of pre-ovipositional embryonic arrest for turtles. How each experimental chapter (Chapters 2-6) addressed my general aim and the specific aims of each chapter is presented in Figure 7.1. A brief summary of the findings presented in each chapter and their implications is presented below.

7.1.1 Latency until breaking of arrest after oviposition (Chapter 2)

Exposure to normoxia after turtle eggs transition from the hypoxic oviducts to the normoxic nest is the trigger that breaks pre-ovipositional embryonic arrest (Kennett *et al.* 1993b; Fordham *et al.* 2006; Fordham *et al.* 2007; Rafferty *et al.* 2013; Rings *et al.* 2015). However, it was not known how long embryos require in normoxia before breaking the arrest. I experimentally identified that 12 to 16 h in normoxia after oviposition is required for green turtle embryos to break embryonic arrest and start active development (Williamson *et al.* 2017a). I found that if eggs are placed back into hypoxia prior to the breaking of arrest (i.e. ≤ 12 h after oviposition) they can be maintained in extended arrest for three days with no reduction in hatching success. However, eggs will not survive to hatching if returned to hypoxia after arrest has broken (> 12 h after oviposition). In conclusion, turtle embryos break from pre-ovipositional embryonic arrest after 12 to 16 h in normoxia but hypoxic incubation before this point can extend arrest without impacting hatching success. Therefore, hypoxic

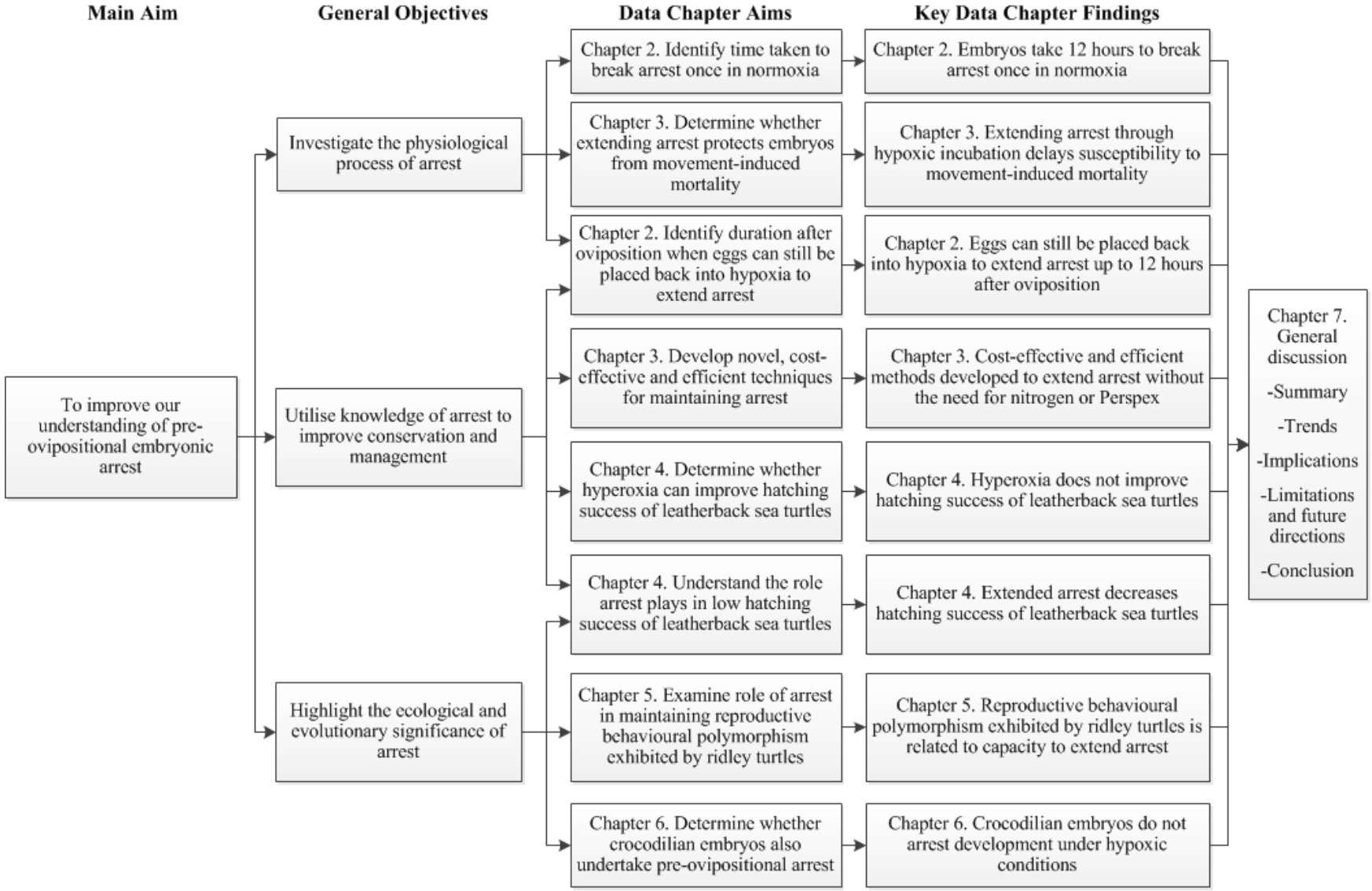


Figure 7.1 Research aims, general structure and key findings for thesis

incubation prior to 12 h after oviposition provides a useful method for researchers to extend embryonic arrest in turtle eggs.

7.1.2 Methods for maintaining eggs in hypoxia to extend embryonic arrest and protect against movement-induced mortality (Chapter 3)

Turtle embryos can suffer from movement-induced mortality when eggs are translocated. For the experiment presented in Chapter 3, I aimed to determine whether extending pre-ovipositional embryonic arrest, by incubating eggs in hypoxia after oviposition, would protect them from movement-induced mortality. Further, I aimed to develop novel, cost-effective and practical methods for maintaining eggs in hypoxia. I demonstrated that extension of pre-ovipositional embryonic arrest after oviposition protects embryos from movement-induced mortality and can be achieved through hypoxic incubation using cost-effective and field-practical methods (Williamson *et al.* 2017b). Vacuum-sealed bags in particular offer a promising tool for conservation and research practitioners, working in difficult field environments, to avoid movement-induced mortality when transporting eggs. The critical advantage associated with the use of vacuum-sealed bags is that they are affordable and remove the need to take gas cylinders into the field.

7.1.3 Impact of arrest on leatherback turtle reproductive success (Chapter 4)

Low hatching success (~50%) and early-stage embryonic mortality is a conservation concern for leatherback turtles (Bell *et al.* 2003; Rafferty *et al.* 2011; Wallace *et al.* 2011; Eckert *et al.* 2012). I aimed to assess whether hyperoxia (32-42% O_2) during the first five days of incubation positively impacted hatching success, and conversely, whether hypoxia (1% O_2) negatively impacted hatching success. I found that hyperoxic incubation did not increase hatching success

or reduce early-stage embryonic mortality of leatherback turtles. However, I demonstrated that hypoxic incubation and subsequent extension of arrest longer than three days negatively impacts hatching success of leatherbacks, which suggests there is a potential trade-off for mothers when delaying nesting. In conclusion, pre-ovipositional embryonic arrest may play an important role in the reproductive success of the leatherback turtle. However, the causes of low hatching success and high levels of early-stage embryonic death remain to be elucidated.

7.1.4 Difference in arrest between arribada and non-arribada olive ridley turtles (Chapter 5)

Olive ridley sea turtles nest in either an aggregated nesting event, termed an 'arribada', or nest solitarily in 'non-arribada' events. The inter-nesting interval differs with the nesting strategy used, with arribada turtles nesting every 28 days and non-arribada turtles nesting every 14 days (Pritchard 1969; Plotkin *et al.* 1997; Kalb 1999; Rostal 2007). I aimed to assess whether embryos that are oviposited during an arribada differ in their capacity to be maintained in pre-ovipositional embryonic arrest when compared with those from a non-arribada nesting event. Importantly, I found that arribada embryos are capable of extending pre-ovipositional arrest for longer than non-arribada embryos. This may be an important physiological difference that allows the maintenance of the behavioural reproductive polymorphism exhibited by olive ridley turtles.

7.1.5 Absence of hypoxia-induced embryonic arrest in crocodilians (Chapter 6)

Whether crocodilians, like turtles, exhibit pre-ovipositional embryonic arrest was unknown and unexamined. I aimed to test whether crocodilian embryos also arrest development in hypoxia after oviposition. Hypoxic incubation of saltwater crocodile eggs failed to delay development (Williamson *et al.* 2017c), in contrast to what has been shown in turtles (Chapters 2-5). This is the first experimental evidence that, unlike turtles, crocodilian embryos are not capable of

arresting development in hypoxia at oviposition. Potentially, this means crocodilians are less able to delay nesting than turtles in order to avoid adverse nesting conditions.

7.2 General trends for how oxygen availability affects embryonic development in crocodilians and turtles

In the following sections I integrate information from each experimental chapter presented in this thesis on how variation in oxygen availability during early-stage embryonic development impacts subsequent development in crocodilians and turtles.

7.2.1 Opaque white spot development

Generally, oxygen availability affects the formation of the opaque white spot on the egg. Incubation of crocodilian and turtle eggs in hypoxia usually results in a delay, equal to time spent in hypoxia, until opaque white spot formation (Chapters 2-6). However, a high proportion of saltwater crocodile eggs did not delay formation of the white spot in hypoxia. These eggs formed a white spot within one or two days, suggesting that the embryos were not arrested (Chapter 6). Hyperoxia has no effect on the timing of white spot development in either turtles or crocodiles (Chapters 4 & 6). Across all experiments, formation of the white spot was not a good indication that eggs would successfully develop to hatching because most eggs formed white spots even in treatments with low or zero hatching success. The findings of the experiments indicate that monitoring white spot formation is a useful proxy for assessing clutch fertilisation rate (Abella *et al.* 2017), because even embryos that died at early developmental stages formed white spots. However, it has limited utility as a marker of successful embyonic development. In conclusion, white spots will eventually form regardless of oxygen availability, whilst hypoxia usually delays

white spot formation and hyperoxia has no detectable impact on latency till formation of the spot.

7.2.2 Incubation period

In eggs that survive to hatching, variation in oxygen availability early in incubation can affect the total duration of incubation. However, although most crocodile eggs died after hypoxic incubation, the single crocodilian egg to hatch after being incubated in hypoxia for three days hatched at approximately the same time as those from the control and hyperoxic treatments (Chapter 6). Again, this provides further evidence that crocodilian eggs are not capable of arresting development in hypoxia. Hypoxic incubation of turtle eggs generally resulted in a delayed hatching date, with the delay being approximately equivalent to the time eggs spent in hypoxia (Chapters 3, 4 and 5). However, olive ridley turtle eggs maintained in arrest for 15 days only increased total incubation period by 7 days (Chapter 5). This difference could be due to eggs compensating for extended periods of arrest by accelerating the rate of development once arrest is broken. Such a response to extended arrest was shown in a species of freshwater turtle (Kennett *et al.* 1998; Fordham *et al.* 2006). Alternatively, the nests containing the 15-day treatment eggs could have been experiencing warmer temperatures as they were incubating later during the nesting season when temperatures are hotter, which could have also accelerated the rate of embryonic development. Embryos may have some form of internal clock that tracks time spent in embryonic arrest. Finally, hyperoxia had no detectable impact on incubation duration (Chapters 4 & 6). In summary, variation in oxygen availability at the start of egg incubation can affect incubation duration, with hypoxic incubation usually resulting in delayed hatching and hyperoxic incubation having no impact.

7.2.3 Hatching success

My results indicate that variation in oxygen availability during the early stages of incubation subsequently affects the likelihood that crocodilian and turtle eggs will hatch. For example, only a single crocodile egg hatched after hypoxic incubation, which again is further evidence for a lack of capacity for crocodilians to arrest embryonic development in hypoxia (Chapter 6). Across my range of experiments, hypoxic incubation of turtle eggs also often resulted in a reduction in hatching success (Chapters 2-5). However, the reduction in hatching success was not as extreme as that shown for crocodilians. The reduction in hatching success was more pronounced with longer periods of hypoxic incubation (Chapter 5). There was also interspecific and between-experiment variation in hatching success of eggs exposed to hypoxia. For example, three-day hypoxic incubation did not reduce hatching success of green sea turtle eggs (Chapter 2) and leatherback turtle eggs (Chapter 4) but it did in olive ridley sea turtle eggs (Chapters 3 & 5). I suggested that this interspecific difference may be attributed to the difference in incubation periods for these species, because three days is proportionally more of the total incubation period for olive ridley turtle eggs than the other species (Chapter 3). Extension of pre-ovipositional arrest for five days through hypoxic incubation in flatback turtles also reduced hatching success (Rings *et al.* 2015). However, hyperoxic incubation of crocodile and turtle eggs had no impact on hatching success (Chapters 4 & 6). This finding correlates with the only similar study which also found no impact of hyperoxic incubation during the first five days of development for flatback turtles (Rings *et al.* 2015). In conclusion, hypoxic incubation (especially for extended periods) during early-stage development is likely to impact hatching success, whilst hyperoxic incubation is not.

7.2.4 Hatchling morphology and fitness

Variation to oxygen availability during early-stage incubation of crocodilian and turtle eggs sometimes affects hatchling morphology and fitness. Hyperoxic incubation had no impact on hatchling morphology and fitness of saltwater crocodiles or leatherback turtles (Chapters 4 & 6). This observation is at odds with what was shown for flatback turtles, where hyperoxic incubation of eggs for five days resulted in larger hatchlings that crawled fasted but swam slower than a control group (Rings *et al.* 2015). Hypoxic incubation usually had no impact upon hatchling morphology and fitness (Chapters 3, 4 & 6). However, we did not assess hatchlings in the experiment described in Chapter 2. Furthermore, after extreme periods of hypoxic incubation (8 – 15 days) olive ridley hatchlings were smaller than those of a control group (Chapter 5). Previous studies involving hypoxic incubation also found impacts on hatchling morphology and fitness (Kennett *et al.* 1998; Fordham *et al.* 2007; Rings *et al.* 2015). Similarly to hatchings success, further investigation of species-specific differences and the impact of the duration of extended arrest on hatchling morphology and fitness is warranted. In conclusion, hypoxic and/or hyperoxic incubation at the start of incubation usually does not impact hatchling morphology or fitness, although, this is not always the case.

7.3 Ecological implications of pre-ovipositional embryonic arrest

The findings from the experiments described in Chapters 2 to 6 highlight important ecological ramifications of the capacity to arrest embryonic development prior to oviposition. The hypoxic treatments in each of these experiments were aimed towards simulating periods of delayed nesting by the mother. That is, our hypoxic experimental treatments were designed so that the availability of oxygen approximated that observed in the oviducts of gravid turtles (Rafferty *et*

al. 2013). The findings show that extended periods of arrest in hypoxia negatively impact hatching success. Therefore, there is a likely ecological trade-off for the mother between delaying nesting to avoid adverse conditions and maximising hatching success.

Pre-ovipositional arrest, in turtles at least, allows the mother greater flexibility in nesting and may lessen the impact of the trade-off with reduced hatching success. However, there still appears to be an optimal window for oviposition because embryos cannot remain arrested indefinitely without a reduction in hatching success (Chapters 4 & 5). There are differences between species in this optimal time as well. Previous research has also shown that freshwater turtles have a greater capacity than sea turtles to avoid adverse conditions through use of pre-ovipositional arrest (Kennett *et al.* 1993a; Kennett *et al.* 1993b; Buhlmann *et al.* 1995; Kennett *et al.* 1998; Fordham *et al.* 2006; Fordham *et al.* 2007; Rafferty *et al.* 2013). In contrast, crocodilians may be less able to avoid adverse conditions than turtles, because we found no evidence for hypoxia-mediated pre-ovipositional arrest (Chapter 6). However, to my knowledge there are no published reports of oxygen availability in the crocodilian oviduct. I attempted to measure the oxygen availability in the oviduct of freshwater crocodiles (*Crocodylus johnstoni*). However, I was unsuccessful in these endeavours (see section 7.5 for more detail). Further studies along this line are warranted, since they will potentially show whether, like the turtle oviduct, the crocodile oviduct is a hypoxic environment.

7.4 Evolution of pre-ovipositional embryonic arrest

It is interesting that even though crocodilians and turtles share similar reproductive and developmental traits, only turtles appear to be capable of pre-ovipositional embryonic arrest. The difference between crocodilians and turtles in the level of parental care provided to the nest and

hatchlings may partially explain the lack of pre-ovipositional embryonic arrest in crocodilians (Chapter 6). Whether pre-ovipositional arrest evolved in turtles or in an ancestral taxon is unknown. How did this remarkable reproductive trait evolve? The simple answer is that we do not know, but it would be very interesting to investigate. Furthermore, whether it is present in other species that exhibit similar developmental patterns, such as tuatara and chameleons, is unknown. Investigation of the evolutionary history and genomic signalling for pre-ovipositional embryonic arrest would be exciting and informative.

7.5 Limitations and directions for future research

During the course of my study there have been many ideas and questions regarding pre-ovipositional embryonic arrest that I have not had the time or resources to address. First, there are some limitations to my experiments. Secondly, there are particular aspects from each experiment that could be investigated further. Finally, there are general avenues for investigation that I was unable to embark upon. The following section outlines some of these experimental limitations and directions for further investigation.

7.5.1 Latency until breaking of arrest after oviposition (Chapter 2)

I was able to identify that, following oviposition, *Chelonia mydas* embryos break arrest after 12 hours of exposure to normoxia. However, this experiment was conducted with all eggs maintained at 28ºC, and there could potentially be a difference in the latency until breaking of arrest if eggs are maintained at different temperatures. Incubation temperature is known to influence the rate of embryonic development in turtles (Yntema 1978; Miller 1982; Ewert 1985; Miller 1985). However, a study conducted on a range of turtles (*C. mydas, Chelodina colliei, Chelodina longicollis,* and *Emydura macquarii*) found no difference in latency until white spot

formation across three different temperatures (24, 28, 32ºC), except for *E. macquarii*, which took more time to form white spots when incubated at 24 ºC (Rafferty and Reina 2014). Future studies should assess whether there is any difference in latency until breaking of arrest under varied incubation temperatures.

There could also be an allometric relationship between egg size and latency until breaking of arrest. Changes in morphological size often result in predictable changes in physiological processes (Calder 1982; Damuth 2001; Gillooly *et al.* 2002). It would also be reasonable to assume that oxygen would take longer to reach the embryo in larger eggs. Furthermore, freshwater turtle eggs differ in shape to marine turtle eggs (oblate spheroid vs sphere), and embryos may be closer to the porous eggshell. Further investigations should also assess how latency until breaking of varies with egg size and shape.

Finally, I only assessed the latency until breaking of arrest in one species of turtle (*C. mydas*). The 12 hours or more of normoxic incubation required to break arrest for this species may be different to how long it takes for other species of turtle, especially in other species that take longer periods of time to form white spots, such as freshwater turtles and leatherback turtles (Chan 1989; Rafferty and Reina 2014). However, observations of multiple species of turtle embryos, both freshwater and marine, found discernible signs of post-ovipositional development 12 to 24 hours after oviposition (Ewert 1985; Miller 1985). More interspecific comparisons of the latency until the breaking of arrest would be useful.

7.5.2 Methods for maintaining eggs in hypoxia to extend embryonic arrest and protect from movement-induced mortality (Chapter 3)

I was able to show that extension of arrest, through hypoxic incubation, protects embryos from movement-induced mortality, and that this can be achieved efficiently and cost-effectively using vacuum-sealed bags. However, improvements could be made to the method for maintaining eggs in hypoxia. For example, specially designed vacuum bags suited for spherical objects would be beneficial to reduce the potential for air pockets remaining when sealing turtle eggs. Furthermore, it has been shown that simply inundating freshwater turtle eggs, by keeping them underwater, is an efficient method for maintaining arrest (Fordham *et al.* 2007). Whether this technique is equally suitable for marine turtles should be investigated.

As mentioned previously, temperature is a major influence on embryonic development in turtles (Yntema 1978; Miller 1982; Ewert 1985; Miller 1985). I did not examine how variation in temperature during hypoxia impacts pre-ovipositional arrest and subsequent embryonic development. Therefore, future studies should examine how temperature variation during hypoxic egg transportation influences development.

In this experiment, I only maintained eggs in arrest for three days. This duration is likely longer than what would ever be required for transportation. However, researchers could want to maintain eggs oviposited on different nights at the same stage of embryonic development, and therefore could benefit from the ability to maintain eggs in hypoxia for longer than three days. Furthermore, I only tested these hypoxic incubation methods on one turtle species (*Lepidochelys olivacea*). Therefore, future studies should examine the influence of the duration of arrest on developmental success and any interspecific differences that exist.

7.5.3 Impact of arrest on leatherback turtle reproductive success (Chapter 4)

I found that extended periods of pre-ovipositional embryonic arrest lowered hatching success for leatherback turtles. Whilst eggs were incubated in hypoxia with the aim to simulate extended egg retention by the mother, the method is not a perfect representation of a mother's oviduct. Therefore, the ecological implications drawn from this finding are limited. There may be potentially important biochemical interactions specific to the oviducal environment that allow the eggs to be maintained in arrest for longer periods of time without negatively impacting subsequent development as much. However, this could potentially be overcome by experimentally preventing females from nesting for consecutive nights and assessing the subsequent impact upon embryonic development.

I also found that hyperoxic incubation (32-42% O_2) did not increase hatching success in leatherback turtles. However, from my experiment we cannot rule out the possibility that smaller or even larger increases in oxygen availability might be beneficial to hatch rate. The increase in oxygen availability (32-42% vs 21%) was quite a large one and high levels of available oxygen can be toxic to vertebrate embryos (Umaoka *et al.* 1992; Karagenc *et al.* 2004). Potentially some level between sea-level atmospheric normoxia (21%) and 32% could have beneficial impacts on leatherback turtle development.

Although I found that extended arrest causes a decrease in leatherback turtle hatching success, I did not identify the cause or causes of their consistently low hatching success. There are many potential causes of their consistently low hatching success and these effects may interact in a complex manner. As summarised in Chapter 4, there are possible negative impacts of abiotic factors, such as oxygen, carbon dioxide, and temperature whilst eggs are incubating in the nest

(Wallace *et al.* 2004; Ralph *et al.* 2005; Santidrián Tomillo *et al.* 2009; Garrett *et al.* 2010). It has been shown hatching success varies significantly with maternal identity (Bell *et al.* 2003; Rafferty *et al.* 2011) and this may be related to maternal physiology (Perrault *et al.* 2012).

Environmental chemical contaminants have been suggested as a possible contributing factor to the low hatching success of leatherback turtles (Bell *et al.* 2003). Indeed, a recent study of the Caribbean Costa Rican population found a relationship between persistent organic pollutants (POPs) found within subsampled eggs and the hatching success of their respective clutch (De Andrés *et al.* 2016). POPs are maternally transferred to the egg and mothers are probably contaminated at their foraging grounds while the ovarian follicles are still forming (Guirlet *et al.* 2010). POPs, such as endocrine-disrupting polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), may disrupt normal embryonic development (Guirlet *et al.* 2010; van de Merwe *et al.* 2010; Stewart *et al.* 2011). Leatherback turtles were found to have ten times greater levels of POPs when compared with green sea turtles (van de Merwe *et al.* 2010; De Andrés *et al.* 2016).

Leatherbacks that had longer nesting re-migration intervals tended to have higher levels of POPs, suggesting that time spent foraging is an important determinant of POP levels (Guirlet *et al.* 2010). It has also been suggested that females may offload POPs into their eggs in order to reduce their own contaminant load (Guirlet *et al.* 2010). De Andrés *et al.* (2016) found high levels of within-clutch variation in POP levels, which may be partially responsible for within-clutch variation in embryonic death. This therefore could be viewed as a potential physiological detoxification pathway that mothers employ to load some eggs with greater levels of contaminants than other eggs, which therefore decreases risk of embryonic death for some of the eggs in each clutch.

Metals or trace elements, both essential and toxic (or non-essential) are maternally transferred to eggs in leatherbacks as well (Guirlet *et al.* 2008). Perrault *et al.* (2011) found that within the Florida (USA) nesting population levels of mercury could be negatively impacting selenium concentrations and that selenium-to-mercury ratio is positively correlated with hatching success. However, the same relationship was not found for the St. Croix nesting population in a study of greater sample size (Perrault *et al.* 2013). As with POPs, reproduction could offer females an opportunity to reduce their toxic metal burden (Burger and Gibbons 1998; Guirlet *et al.* 2008). Contaminants within the nesting substrate could also be assimilated into the albumin and yolk through the permeable eggshell whilst eggs are incubating in the nest and this could disrupt normal embryonic development (Marco *et al.* 2004). The possibility that leatherbacks accumulate more contaminants or are more susceptible to developmental failure as a result of contamination merits investigation and remediation urgently in light of the perilous conservation status of many populations of this species. Overall, it is clear the reason for the high incidence of embryonic death, especially at early embryonic stages, remains elusive to researchers and still warrants further investigation.

7.5.4 Difference in arrest between arribada and non-arribada olive ridleys (Chapter 5)

My results from Chapter 5 support the conclusion that arribada nesting females produce eggs that are capable of being maintained in pre-ovipositional embryonic arrest for longer than eggs produced by non-arribada nesting females. There have been observations that turtles nesting in arribadas generally have a 28-day inter-nesting interval compared to only 14-days for non-arribada nesters (Plotkin *et al.* 1997; Kalb 1999; Rostal 2007). One limitation to my study is that I cannot be sure of the inter-nesting interval of the mothers from which I collected eggs. Therefore, I cannot rule out the possibility that the non-arribada females may have simply been

late or early for an arribada. This could be addressed by performing ultrasound on nesting females to check if it is their first or second clutch, and then only collecting from females if it is their first. It would also be instructive to attach satellite tags to females who's eggs are collected to identify if they subsequently nested again in an arribada or in a solitary event. This would also allow for assessment of whether the turtles nesting during an arribada repeat this behaviour for their second nest of the season or if they switch to nesting solitarily.

Another limitation was that I only compared eggs from arribada and non-arribada nesters that had been laid at Ostional. The relationship between arribada behaviour and pre-ovipositional embryonic arrest could be further elucidated by comparing the capacity to arrest for eggs oviposited at beaches where only solitary nesting occurs with the capacity to arrest in eggs oviposited at arribada beaches (both during and outside of arribadas, as I did at Ostional). Furthermore, confirmation of the importance of pre-ovipositional arrest to arribada behaviour could be garnered by investigation of the capacity for arrested development in Kemp's ridley sea turtle (*Lepidochelys kempii*) eggs.

Finally, as there was an apparent difference in development of eggs oviposited during the two differing nesting tactics, variation in maternal health parameters should be investigated. Haematological, oviducal and ovarian parameters may have a relationship with the ability of the mother's eggs to remain in arrest. This could also subsequently impact upon hatchling health parameters. I did investigate differences in hatchling morphology and fitness between eggs laid during an arribada and non-arribada event. However, studies with larger clutch sample sizes are warranted.

7.5.5 Hypoxia induced embryonic arrest in crocodilians (Chapter 6)

The findings of this experiment indicate that crocodilians are unable to arrest embryonic development in hypoxia unlike turtles. However, it remains unknown whether the level of hypoxia used for my experiment (1% O_2) is actually representative of the level of oxygen available in the crocodilian oviduct. As previously mentioned, investigation of crocodilian *in utero* PO_2 is warranted and I attempted to do this. However, I was unsuccessful. I collaborated with a team of researchers to capture 10 female freshwater crocodiles (*Crocodylus johnstoni*) during the start of the nesting season at Windjana Gorge, Western Australia. However, we were unable to identify any gravid females through inguinal palpation and ultrasound. We attempted to insert a fibre optic oxygen sensing probe into the oviduct of four of the females. However, the veterinarian, T. Scheelings, who performed the same procedure for the only similar study conducted on turtles (Rafferty *et al.* 2013), was unable to locate the probe into the oviduct. Furthermore, whilst conducting my experiment detailed in Chapter 6, I was unable to perform any *in utero* PO_2 measurements on the captive saltwater crocodiles (*Crocodylus porosus*) at Crocodylus Park. This was because of the value of reproductive females to the owner and potential for health complications when capturing and restraining gravid saltwater crocodiles, caused by eggs breaking in the oviduct.

During my time at Crocodylus Park there were two reproductive females that were killed by their male breeding partner also enclosed in their pens. From these two females I was able to dissect the relatively fresh reproductive tracts and preserve them for morphological analysis. I conducted MRI and synchrotron micro-CT scans of the organs. These scans are currently being analysed. Therefore, I hope to report on the reproductive morphology of saltwater crocodiles in the future. One interesting finding from this study was that saltwater crocodiles appear to have an extremely

complex muscular vagina, possibly with two sphincters. This may explain why we had difficulty in placing the oxygen probe into the oviduct of the freshwater crocodiles at Windjana Gorge. Further investigation of the morphological differences between crocodilian and turtle oviducts could be useful in understanding the difference in embryonic development exhibited by them.

As mentioned in Chapter 6, another possible limitation to the study was delay between oviposition and placement of eggs into their respective treatment. As discussed in Chapter 6, I think the delay is unlikely to have influenced the outcome of the experiment. Furthermore, I am currently not aware of any potential method to shorten the delay. Conceivably, a specifically designed pen that allowed close access to the eggs whilst protecting the researcher from the mother could help to reduce the delay between oviposition and placement of eggs in hypoxia. Alternatively, a methodology could be developed to flood the nest chamber immediately after oviposition and create a hypoxic incubation environment for the eggs. Such a methodology would also be more ecologically relevant, because crocodilian nests commonly experience inundation during incubation of eggs.

I also only conducted this study using captive laid eggs. Eggs laid by non-captive crocodilians would be optimal for this experiment. However, there would be immense logistical issues of waiting by crocodilian nests in the field, for possibly weeks at a time, for mothers to nest. Finally, as with my studies conducted on turtles, I only examined the response to hypoxic incubation in one species of crocodilian. Again, future studies could examine any interspecific differences in embryonic development when eggs are incubated in hypoxia.

7.5.6 General limitations and directions for future research

In general, more experimentation with hypoxic incubation on a range of freshwater and sea turtle species will help to identify species-specific differences in the capacity to arrest and allow inferences on how this may be relevant to the ecology and evolutionary history of individual species. As previously mentioned, investigation of whether taxa other than turtles exhibit pre-ovipositional arrest and how the arrest functions in those other taxa is warranted. Good candidate species for investigating pre-ovipositional embryonic arrest are tuatara, chameleons and some other squamates (Andrews *et al.* 2008; Rafferty and Reina 2012). If arrest occurs in the oviducts of other taxa it is most likely that oxygen availability is the controlling variable (Andrews 2002; Rafferty *et al.* 2013) and therefore simple hypoxic incubation experiments would be useful. However, measurement of the oviducal oxygen availability for taxa other than turtles is needed. Again, focus should be placed on other reptiles because of the diverse range of developmental patterns that they exhibit (Andrews and Mathies 2000; Andrews 2004).

From this thesis, and other studies (Kennett *et al.* 1993b; Kennett *et al.* 1998; Fordham *et al.* 2006; Fordham *et al.* 2007; Rafferty *et al.* 2013; Rings *et al.* 2015), we now have a large body of evidence that implicates oxygen availability in the functioning of embryonic arrest in turtles. However, there is still little information available as to specific molecular mechanisms that arrest development and trigger it to recommence again. Investigation of the regulation of insulin-like growth factor binding proteins during hypoxic incubation is warranted, because they have been suggested to play an important role in the control of pre-ovipositional arrest and embryonic development (Rafferty and Reina 2012). Interspecific comparisons of egg chemistry may enable elucidation of important differences that correlate with the species-specific capacity to extended pre-ovipositional embryonic arrest.

Finally, my studies demonstrate the utility of pre-ovipositional embryonic arrest to conservationists and managers (Chapters 2, 3 and 4). However, further methodological testing would be beneficial to improve our ability to maintain embryonic arrest with minimal impact on hatching success and resultant hatchlings. Furthermore, during all of our hypoxia experiments eggs were maintained at a relatively stable temperature that was within the tolerance range of embryos for development to occur. However, temperature during transport is likely to fluctuate if only vacuum sealed bags are used with no insulation. Therefore, investigation of how fluctuating temperature during maintenance of arrest affects subsequent development will be useful.

7.6 Conclusion

In turtles, pre-ovipositional embryonic arrest breaks after 12 hours of exposure to normoxia which coincides with the start of the embryo's susceptibility to movement-induced mortality. Turtle eggs can be maintained in hypoxia using simple and cost-effective vacuum-sealed bags to prolong pre-ovipositional arrest and protect embryos from movement-induced mortality. Extended pre-ovipositional embryonic arrest negatively impacts hatching success in leatherback turtles. The capacity for extension of pre-ovipositional embryonic arrest in olive ridley turtle eggs may correspond to nesting behaviour, with arribada laid eggs possibly capable of maintaining arrest for longer than solitarily laid ones. Finally, crocodilians, unlike turtles, do not appear to exhibit hypoxia-mediated pre-ovipositional embryonic arrest. The findings of the experiments described in this thesis have contributed new knowledge regarding the function, ecological and evolutionary significance, of pre-ovipositional embryonic arrest (Figure 7.1).

7.7 References

Abella, E, García-Cerdá, R, Marco, A (2017) Non-invasive monitoring of the fertilization rate in sea turtle nests: comparison between two techniques. *Basic and Applied Herpetology* **31**, in press.

Andrews, RM (2002) Low oxygen: A constraint on the evolution of viviparity in reptiles. *Physiological and Biochemical Zoology* **75**, 145-154.

Andrews, RM (2004) Patterns of embryonic development. In 'Reptilian Incubation: Environment, Evolution and Behaviour.' (Ed. DC Deeming.) pp. 75- 102. (Nottingham University Press: United Kingdom)

Andrews, RM, Diaz‐Paniagua, C, Marco, A, Portheault, A (2008) Developmental arrest during embryonic development of the common chameleon (*Chamaeleo chamaeleon*) in Spain. *Physiological and Biochemical Zoology* **81**, 336-344.

Andrews, RM, Mathies, T (2000) Natural history of reptilian development: Constraints on the evolution of viviparity. *BioScience* **50**, 227-238.

Bell, BA, Spotila, JR, Paladino, FV, Reina, RD (2003) Low reproductive success of leatherback turtles, *Dermochelys coriacea*, is due to high embryonic mortality. *Biological Conservation* **115**, 131-138.

Buhlmann, K, Lynch, T, Whitfield Gibbons, J, Greene, J (1995) Prolonged egg retention in the turtle *Deirochelys reticularia* in South Carolina. *Herpetologica* **51**, 457-462.

Burger, J, Gibbons, JW (1998) Trace elements in egg contents and egg shells of slider turtles (*Trachemys scripta*) from the Savannah River Site. *Archives of Environmental Contamination and Toxicology* **34**, 382-386.

Calder, WA (1982) The pace of growth: An allometric approach to comparative embryonic and post embryonic growth. *Journal of Zoology* **198**, 215-225.

Chan, E-H (1989) White spot development, incubation and hatching success of leatherback turtle (*Dermochelys coriacea*) eggs from Rantau Abang, Malaysia. *Copeia* **1989**, 42-47.

Damuth, J (2001) Scaling of growth: Plants and animals are not so different. *Proceedings of the National Academy of Sciences* **98**, 2113-2114.

De Andrés, E, Gómara, B, González-Paredes, D, Ruiz-Martín, J, Marco, A (2016) Persistent organic pollutant levels in eggs of leatherback turtles (*Dermochelys coriacea*) point to a decrease in hatching success. *Chemosphere* **146**, 354-361.

Eckert, KL, Wallace, BP, Frazier, JG, Eckert, SA, Pritchard, PCH, 2012. Synopsis of the biological data on the leatherback sea turtle (*Dermochelys coriacea*). U.S. Department of Interior, Fish and Wildlife Service, Washington, D.C.

Ewert, MA (1985) Embryology of turtles. In 'Biology of the Reptilia.' (Eds C Gans, F Billett, PFA Manderson.) Vol. 14 pp. 75-267. (John Wiley & Sons: New York)

Fordham, D, Georges, A, Corey, B (2006) Compensation for inundation-induced embryonic diapause in a freshwater turtle: Achieving predictability in the face of environmental stochasticity. *Functional Ecology* **20**, 670-677.

Fordham, DA, Georges, A, Corey, B (2007) Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: Science in support of an indigenous enterprise. *Aquaculture* **270**, 105-114.

Garrett, K, Wallace, BP, Garner, J, Paladino, FV (2010) Variations in leatherback turtle nest environments: Consequences for hatching success. *Endangered Species Research* **11**, 147-155.

Gillooly, JF, Charnov, EL, West, GB, Savage, VM, Brown, JH (2002) Effects of size and temperature on developmental time. *Nature* **417**, 70-73.

Guirlet, E, Das, K, Girondot, M (2008) Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea*) of French Guiana. *Aquatic Toxicology* **88**, 267-276.

Guirlet, E, Das, K, Thomé, JP, Girondot, M (2010) Maternal transfer of chlorinated contaminants in the leatherback turtles, *Dermochelys coriacea*, nesting in French Guiana. *Chemosphere* **79**, 720-726.

Kalb, HJ (1999) Behavior and physiology of solitary and arribada nesting olive ridley sea turtles (*Lepidochelys olivacea*) during the internesting period. Texas A&M University.

Karagenc, L, Sertkaya, Z, Ciray, N, Ulug, U, Bahçeci, M (2004) Impact of oxygen concentration on embryonic development of mouse zygotes. *Reproductive BioMedicine Online* **9**, 409-417.

Kennett, R, Christian, K, Bedford, G (1998) Underwater nesting by the Australian freshwater turtle *Chelodina rugosa*: effect of prolonged immersion and eggshell thickness on incubation period, egg survivorship, and hatchling size. *Canadian Journal of Zoology* **76**, 1019-1023.

Kennett, R, Christian, KA, Pritchard, D (1993a) Underwater nesting by the tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*). *Australian Journal of Zoology* **41**, 47-52.

Kennett, R, Georges, A, Palmerallen, M (1993b) Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata: Chelidae*), from northern Australia. *Australian Journal of Zoology* **41**, 37-45.

Marco, A, López-vicente, M, Pérez-mellado, V (2004) Arsenic uptake by reptile flexible-shelled eggs from contaminated nest substrates and toxic effect on embryos. *Bulletin of Environmental Contamination and Toxicology* **72**, 983-90.

Miller, JD (1982) Development of marine turtles. University of New England.

Miller, JD (1985) Embryology of marine turtles. In 'Biology of the reptilia.' (Eds C Gans, F Billett, PFA Maderson.) Vol. 14 pp. 269-328. (John Wiley & Sons: New York)

Perrault, J, Wyneken, J, Thompson, LJ, Johnson, C, Miller, DL (2011) Why are hatching and emergence success low? Mercury and selenium concentrations in nesting leatherback sea turtles (*Dermochelys coriacea*) and their young in Florida. *Marine Pollution Bulletin* **62**, 1671-1682.

Perrault, JR, Miller, DL, Eads, E, Johnson, C, Merrill, A, Thompson, LJ, Wyneken, J (2012) Maternal health status correlates with nest success of leatherback sea turtles (*Dermochelys coriacea*) from Florida. *PLoS One* **7**, e31841.

Perrault, JR, Miller, DL, Garner, J, Wyneken, J (2013) Mercury and selenium concentrations in leatherback sea turtles (*Dermochelys coriacea*): Population comparisons, implications for reproductive success, hazard quotients and directions for future research. *Science of The Total Environment* **463–464**, 61-71.

Plotkin, PT, Rostal, DC, Byles, RA, Owens, DW (1997) Reproductive and developmental synchrony in female *Lepidochelys olivacea*. *Journal of Herpetology* **31**, 17-22.

Pritchard, PCH (1969) The survival status of ridley sea-turtles in American waters. *Biological Conservation* **2**, 13-17.

Rafferty, AR, Evans, RG, Scheelings, FT, Reina, RD (2013) Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. *The American Naturalist* **181**, 245-253.

Rafferty, AR, Reina, RD (2012) Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. *Proceedings of the Royal Society B: Biological Sciences* **279**, 2299-2308.

Rafferty, AR, Reina, RD (2014) The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. *Journal of Experimental Marine Biology and Ecology* **450**, 91-97.

Rafferty, AR, Santidrián Tomillo, P, Spotila, JR, Paladino, FV, Reina, RD (2011) Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). *PLoS One* **6**, e21038.

Ralph, CR, Reina, RD, Wallace, BP, Sotherland, PR, Spotila, JR, Paladino, FV (2005) Effect of egg location and respiratory gas concentrations on developmental success in nests of the leatherback turtle, *Dermochelys coriacea*. *Australian Journal of Zoology* **53**, 289-294.

Rings, CC, Rafferty, AR, Guinea, ML, Reina, RD (2015) The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. *Physiological and Biochemical Zoology* **88**, 116-127.

Rostal, DC (2007) Reproductive physiology of the ridley sea turtle. In 'Biology and conservation of ridley sea turtles.' (Ed. PT Plotkin.) pp. 151-165. (Johns Hopkins University Press: Baltimore, Maryland)

Santidrián Tomillo, P, Suss, JS, Wallace, BP, Magrini, KD, Blanco, G, Paladino, FV, Spotila, JR (2009) Influence of emergence success on the annual reproductive output of leatherback turtles. *Marine Biology* **156**, 2021-2031.

Stewart, KR, Keller, JM, Templeton, R, Kucklick, JR, Johnson, C (2011) Monitoring persistent organic pollutants in leatherback turtles (*Dermochelys coriacea*) confirms maternal transfer. *Marine Pollution Bulletin* **62**, 1396-1409.

Umaoka, Y, Noda, Y, Narimoto, K, Mori, T (1992) Effects of oxygen toxicity on early development of mouse embryos. *Molecular Reproduction and Development* **31**, 28-33.

van de Merwe, JP, Hodge, M, Whittier, JM, Ibrahim, K, Lee, SY (2010) Persistent organic pollutants in the green sea turtle *Chelonia mydas*: nesting population variation, maternal transfer, and effects on development. *Marine Ecology Progress Series* **403**, 269-278.

Wallace, BP, DiMatteo, AD, Bolten, AB, Chaloupka, MY, Hutchinson, BJ, Abreu-Grobois, FA, Mortimer, JA, Seminoff, JA, Amorocho, D, Bjorndal, KA, Bourjea, J, Bowen, BW, Briseño Dueñas, R, Casale, P, Choudhury, BC, Costa, A, Dutton, PH, Fallabrino, A, Finkbeiner, EM, Girard, A, Girondot, M, Hamann, M, Hurley, BJ, López-Mendilaharsu, M, Marcovaldi, MA, Musick, JA, Nel, R, Pilcher, NJ, Troëng, S, Witherington, B, Mast, RB (2011) Global conservation priorities for marine turtles. *PLoS One* **6**, e24510.

Wallace, Bryan P, Sotherland, Paul R, Spotila, James R, Reina, Richard D, Franks, Bryan F, Paladino, Frank V (2004) Biotic and abiotic factors affect the nest environment of embryonic leatherback turtles, *Dermochelys coriacea*. *Physiological and Biochemical Zoology* **77**, 423-432.

Williamson, SA, Evans, RG, Reina, RD (2017a) When is embryonic arrest broken in turtle eggs? *Physiological and Biochemical Zoology* **90**, 523-532.

Williamson, SA, Evans, RG, Robinson, NJ, Reina, RD (2017b) Hypoxia as a novel method for preventing movement-induced moratlity during translocation of turtle eggs. *Biological Conservation* **216**, 86-92.

Williamson, SA, Evans, RG, Manolis, SC, Webb, GJ, Reina, RD (2017c) Ecological and evolutionary significance of a lack of capacity for extended developmental arrest in crocodilian eggs. *Royal Society Open Science* **4**, 171439.

Yntema, CL (1978) Incubation times for eggs of the turtle *Chelydra serpentina* (*Testudines: Chelydridae*) at various temperatures. *Herpetologica* **34**, 274-277.

Appendices

Appendix A. Williamson *et al.* (2017) *Physiological and Biochemical Zoology publication*

PDF

When Is Embryonic Arrest Broken in Turtle Eggs?

Sean A. Williamson[1,*]
Roger G. Evans[2]
Richard D. Reina[1]
[1]School of Biological Sciences, Monash University, Clayton, Victoria, Australia; [2]Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton, Victoria, Australia

Accepted 4/14/2017; Electronically Published 6/21/2017

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.

*Corresponding author; e-mail: [redacted]

Physiological and Biochemical Zoology 90(5):523–532. 2017. © 2017 by The University of Chicago. All rights reserved. 1522-2152/2017/9005-6172$15.00. DOI: 10.1086/692630

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

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).

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

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

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

	Control	.5 h	1 h	2 h	4 h	8 h	12 h	16 h	24 h	48 h
Mass (g)	46.8 ± .8	46.4 ± .9	46.7 ± .9	47.4 ± .8	48.1 ± .9	47.0 ± .8	46.5 ± .9	46.6 ± .8	47.9 ± 1.0	47.4 ± 1.0
Diameter (mm)	43.9 ± .3	43.9 ± .3	43.9 ± .3	44.2 ± .3	43.9 ± .3	43.5 ± .3	43.7 ± .3	43.5 ± .3	43.9 ± .3	43.9 ± .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

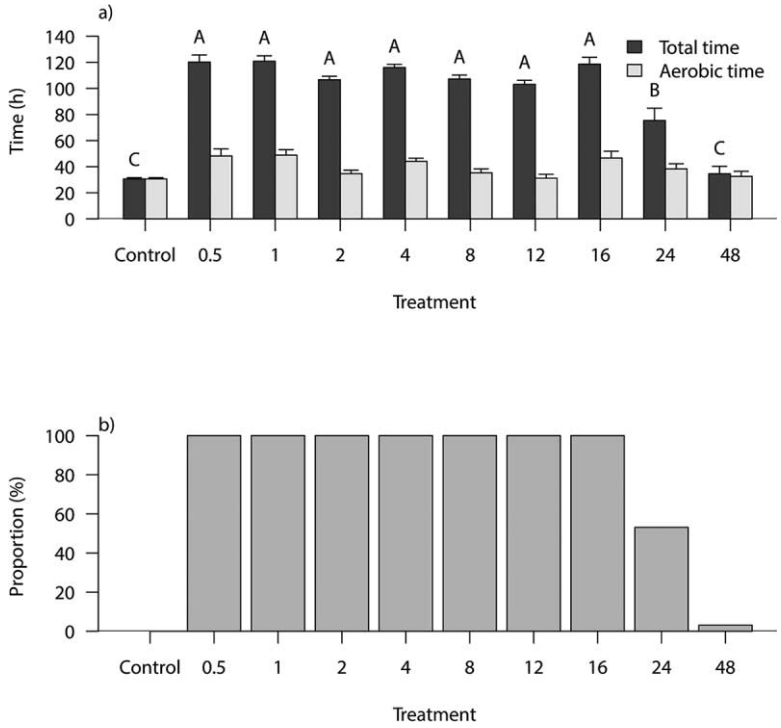


Figure 1. Latency after oviposition until formation of the white spot on eggs (*a*) and proportion of white spots to form after hypoxia in each treatment group (*b*). Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Data in *a* are means ± SE of $n = 31–37$ per 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; $P < 0.05$).

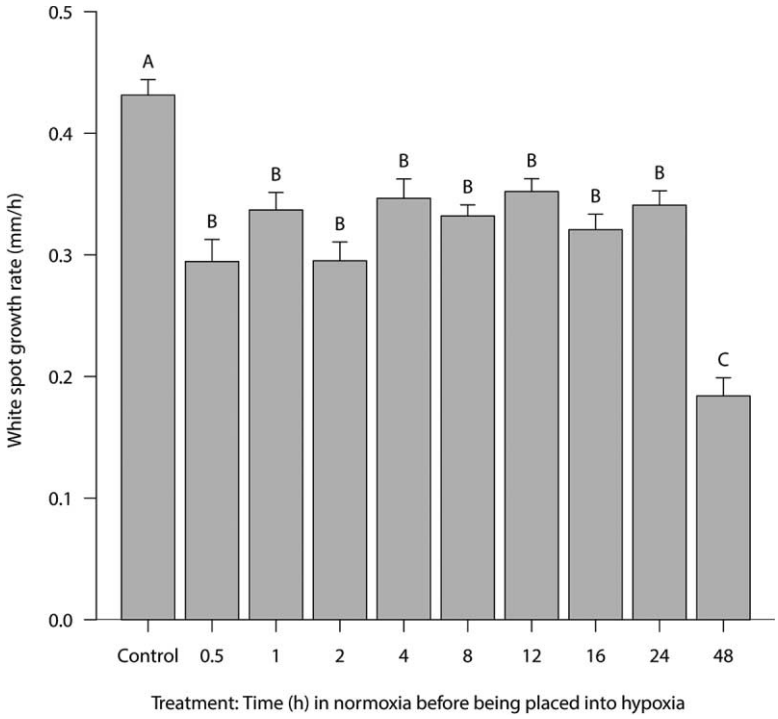


Figure 2. Rate of growth of the white spot on eggs in each treatment group after eggs were removed from hypoxia. Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Data are means ± SE of $n = 25–37$. Similar letters indicate that the white spot growth rate did 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.

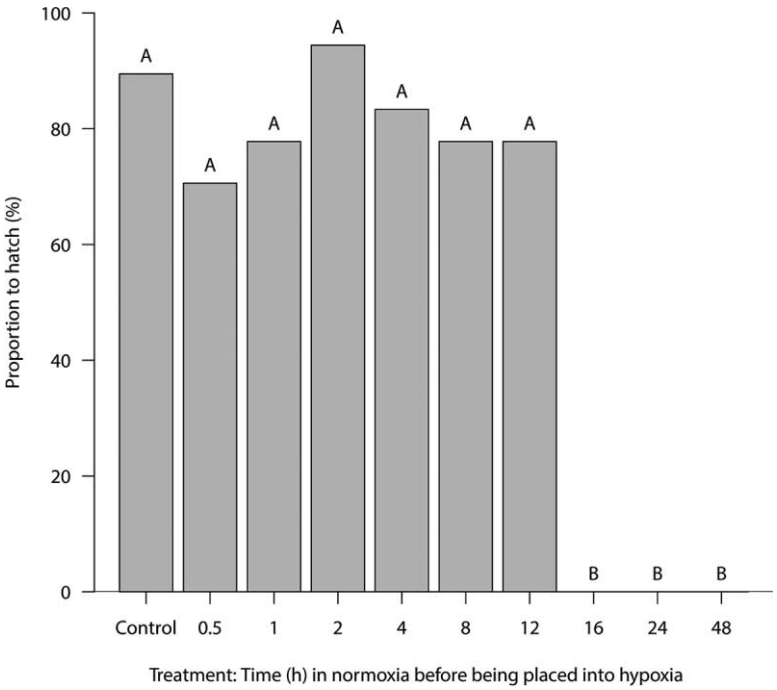


Figure 3. Hatching success (proportion of eggs to hatch) in each treatment where eggs were placed into hypoxia for 3 d after differing amounts of time in normoxia ($n = 16$–20). Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Similar letters indicate that the 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

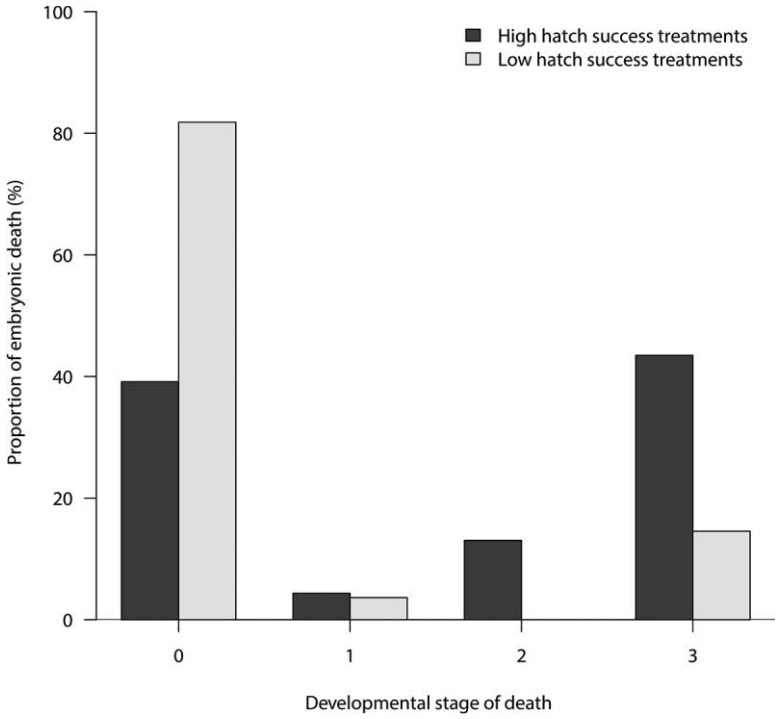


Figure 4. Proportion of embryos from the first collection night that died at each developmental stage. Eggs were placed into hypoxia for 3 d at various time points (0.5–48 h) after oviposition. Dark gray bars indicate treatments with high hatching success (control and 0.5–12-h treatments; $n = 23$), 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-

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.

Acknowledgments

We thank B. Holsworth and the Holsworth Wildlife Research Endowment for generous financial support. Financial support to S.A.W. was made possible through an Australian Postgraduate Award from the Australian Research Council. Thank you to J. Guy from Good Food Solutions and his family for generously catering for our stay on the island. Thanks also to C. Rings and T. Rafferty for their support during the project. We are grateful to D. Annese, E. Perkins, and M. Roberts at the Heron Island Research Station for assisting us while we completed the study. Thanks to J. Ferguson and the Queensland National Parks Turtle Volunteers for their assistance in the field. Finally, a heartfelt thank you in particular to C. Neilson for continual support and volunteering over multiple weeks to help with data collection at all hours of the night.

Literature Cited

Andrews R.M. 2002. Low oxygen: a constraint on the evolution of viviparity in reptiles. Physiol Biochem Zool 75:145–154.

Andrews R.M. and T. Mathies. 2000. Natural history of reptilian development: constraints on the evolution of viviparity. Bioscience 50:227–238.

Aubret F., G. Blanvillain, and P.J.R. Kok. 2015. Myth busting? effects of embryo positioning and egg turning on hatching success in the water snake *Natrix maura*. Sci Rep 5:13385.

Beggs K., J. Young, A. Georges, and P. West. 2000. Ageing the eggs and embryos of the pig-nosed turtle, *Carettochelys insculpta* (Chelonia: Carettochelydidae), from northern Australia. Can J Zool 78:373–392.

Booth D.T. 2000. Incubation of eggs of the Australian broad-shelled turtle, *Chelodina expansa* (Testudinata: Chelidae), at different temperatures: effects on pattern of oxygen consumption and hatchling morphology. Aust J Zool 48:369–378.

Buhlmann K., T. Lynch, J. Whitfield Gibbons, and J. Greene. 1995. Prolonged egg retention in the turtle *Deirochelys reticularia* in South Carolina. Herpetologica 51:457–462.

Deeming C.D. 1991. Reasons for the dichotomy in egg turning in birds and reptiles. Pp. 307–324 in D.C. Deeming and M.W.J. Ferguson, eds. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge.

Deeming C.D. and M.B. Thompson. 1991. Gas exchange across reptilian eggshells. Pp. 277–284 in D.C. Deeming and M.W.J. Ferguson, eds. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge.

Ewert M.A. 1985. Embryology of turtles. Pp. 75–267 in C. Gans, F. Billett, and P.F.A. Manderson, eds. Biology of the Reptilia. Vol. 14. Wiley, New York.

———. 1991. Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. Pp. 173–191 in D.C. Deeming and M.W.J. Ferguson, eds. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge.

Hamann M., M. Godfrey, J. Seminoff, K. Arthur, P. Barata, K. Bjorndal, A. Bolten, et al. 2010. Global research priorities for sea turtles: informing management and conservation in the 21st century. Endanger Species Res 11:245–269.

Kennett R., A. Georges, and M. Palmer-Allen. 1993. Early developmental arrest during immersion of eggs of a tropical freshwater turtle, *Chelodina rugosa* (Testudinata: Chelidae) from Northern Australia. Aust J Zool 41:37–45.

Leslie A.J., D.N. Penick, J.R. Spotila, and F.V. Paladino. 1996. Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990–1991. Chelonian Conserv Biol 2:159–168.

Licht P. 1980. Evolutionary and functional aspects of pituitary gonadotropins in the green turtle, *Chelonia mydas*. Am Zool 20:565–574.

———. 1982. Endocrine patterns in the reproductive cycle of turtles. Herpetologica 38:51–61.

Licht P., D.W. Owens, K. Cliffton, and C. Penaflores. 1982. Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, *Lepidochelys olivacea*. Gen Comp Endocrinol 48:247–253.

Limpus C.J., V. Baker, and J.D. Miller. 1979. Movement induced mortality of loggerhead eggs. Herpetologica 35:335–338.

Marcellini D.L. and S.W. Davis. 1982. Effects of handling on reptile egg hatching. Herpetol Rev 13:43–44.

Miller J.D. 1985. Embryology of marine turtles. Pp. 269–328 in C. Gans and F. Billett, eds. Biology of the Reptilia. Vol. 14. Wiley, New York.

Miller J.D. and C.J. Limpus. 1983. A method for reducing movement-induced mortality in turtle eggs. Mar Turt Newsl 26:10–11.

Owens D. and Y.A. Morris. 1985. The comparative endocrinology of sea turtles. Copeia 1985:723–735.

Parker S.L. and R.M. Andrews. 2006. Evolution of viviparity in sceloporine lizards: in utero P_{O_2} as a developmental constraint during egg retention. Physiol Biochem Zool 79:581–592.

Parker S.L., C.R. Murphy, and M.B. Thompson. 2010. Uterine angiogenesis in squamate reptiles: implications for the evolution of viviparity. Herpetol Conserv Biol 5:330–334.

Parmenter C.J. 1980. Incubation of the eggs of the green sea turtle, *Chelonia mydas*, in Torres Strait, Australia: the effect of movement on hatchability. Aust Wildl Res 7:487–491.

Plotkin P.T., D.C. Rostal, R.A. Byles, and D.W. Owens. 1997. Reproductive and developmental synchrony in female *Lepidochelys olivacea*. J Herpetol 31:17–22.

Rafferty A.R., R.G. Evans, F.T. Scheelings, and R.D. Reina. 2013. Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. Am Nat 181:245–253.

Rafferty A.R. and R.D. Reina. 2012. Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. Proc R Soc B 279:2299–2308.

Rafferty A.R., P. Santidrián Tomillo, J.R. Spotila, F.V. Paladino, and R.D. Reina. 2011. Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). PLoS One 6:1–7.

R Development Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/.

Rings C.C., A.R. Rafferty, M.L. Guinea, and R.D. Reina. 2015. The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. Physiol Biochem Zool 88:116–127.

Rusli M.U., D.T. Booth, and J. Joseph. 2016. Synchronous activity lowers the energetic cost of nest escape for sea turtle hatchlings. J Exp Biol 219:1505–1513.

Santos R.G., H.T. Pinheiro, A.S. Martins, P. Riul, S.C. Bruno, F.J. Janzen, and C.C. Ioannou. 2016. The anti-predator role of within-nest emergence synchrony in sea turtle hatchlings. Proc R Soc B 283:1–7.

Shine R. 1985. The evolution of viviparity in reptiles: an ecological analysis. Pp. 605–694 in C. Gans and F. Billett, eds. Biology of the Reptilia. Vol. 15. Wiley, New York.

Shine R. and L.J. Guillette Jr. 1988. The evolution of viviparity in reptiles: a physiological model and its ecological consequences. J Theor Biol 132:43–50.

Spencer R.-J., M.B. Thompson, and P.B. Banks. 2001. Hatch or wait? a dilemma in reptilian incubation. Oikos 93:401–406.

Thompson M.B. 1985. Functional significance of the opaque white patch in eggs of *Emydura macquarii*. Pp. 387–395 in G. Grigg, R. Shine, and H. Ehmann, eds. Biology of Australasian frogs and reptiles. Surrey Beatty, Chipping Norton, New South Wales.

———. 1993. Oxygen consumption and energetics of development in eggs of the leatherback turtle, *Dermochelys coriacea*. Comp Biochem Physiol A 104:449–453.

Warner D.A., M.A. Moody, and R.S. Telemeco. 2011. Is water uptake by reptilian eggs regulated by physiological processes of embryos or a passive hydraulic response to developmental environments? Comp Biochem Physiol A 160:421–425.

Webb G.J.W., S.C. Manolis, and R. Buckworth. 1983*a*. *Crocodylus johnstoni* in the McKinlay River area, N.T. VI. Nesting biology. Wildlife Res 10:607–637.

Webb G.J.W., S.C. Manolis, R. Buckworth, and G.C. Sack. 1983*b*. An examination of *Crocodylus porosus* nests in two northern Australian freshwater swamps, with an analysis of embryo mortality. Wildl Res 10:571–605.

Webb G.J.W., C.S. Manolis, K.E. Dempsey, and P.J. Whitehead. 1987*a*. Crocodilian eggs: a function overview. Pp. 417–422 in G.J.W. Webb, C.S. Manolis, and P.J. Whitehead, eds. Wildlife management; crocodiles and alligators. Surrey Beatty, Chipping Norton, New South Wales.

Webb G.J.W., S.C. Manolis, P.J. Whitehead, and K. Dempsey. 1987*b*. The possible relationship between embryo orientation opaque banding and the dehydration of albumen in crocodile eggs. Copeia 1987:252–257.

Yntema C.L. 1968. A series of stages in the embryonic development of *Chelydra serpentina*. J Morphol 125:219–252.

Appendix B. Williamson *et al.* (2017) *Biological Conservation publication* PDF

Biological Conservation 216 (2017) 86–92

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

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

Sean A. Williamson[a,*], Roger G. Evans[b], Nathan J. Robinson[c,d,1], Richard D. Reina[a]

[a] School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia
[b] Cardiovascular Disease Program, Biosciences Discovery Institute and Department of Physiology, Monash University, Clayton, VIC 3800, Australia
[c] The Leatherback Trust, Goldring-Gund Marine Biology Station, Playa Grande, Costa Rica
[d] Department of Biology, Indiana-Purdue University, Fort Wayne, IN, USA

ARTICLE INFO

Keywords:
Embryonic development
Turtle conservation
Hypoxia
Egg relocation
Embryonic arrest
Olive ridley

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

* *Corresponding author.
E-mail addresses: [redacted] (S.A. Williamson).
[1] Present address: Cape Eleuthera Institute, Eleuthera, The Bahamas.

http://dx.doi.org/10.1016/j.biocon.2017.10.009
Received 17 June 2017; Received in revised form 14 September 2017; Accepted 9 October 2017
0006-3207/ © 2017 Elsevier Ltd. All rights reserved.

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 ($p = 0.14$,

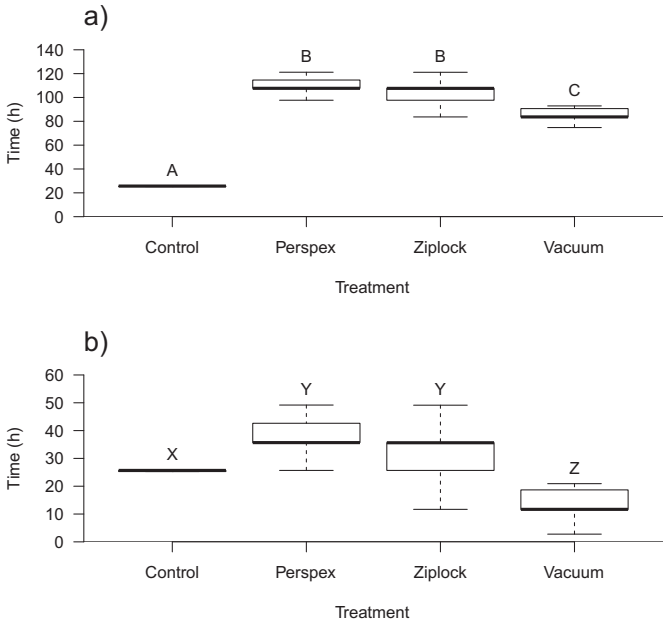


Fig. 2. Latency till formation of the white spot on eggs measured as a) total time, or b) aerobic time. Eggs were subjected to either a control (normoxic) treatment ($n = 76$), or placed into Perspex chambers ($n = 74$) or ziplock bags ($n = 70$) into which nitrogen gas was pumped to exclude oxygen, 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; $p \leq 0.05$).

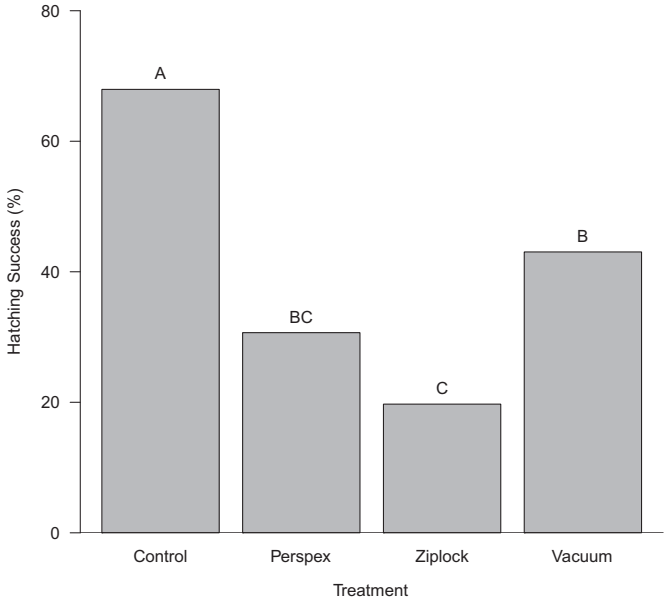


Fig. 3. Total hatching success for olive ridley eggs placed into four treatments. Eggs were placed into either a control treatment ($N = 78$), Perspex chambers ($N = 75$) or ziplock bags ($N = 71$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed plastic bags ($N = 79$). When letters 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; $p \leq 0.05$).

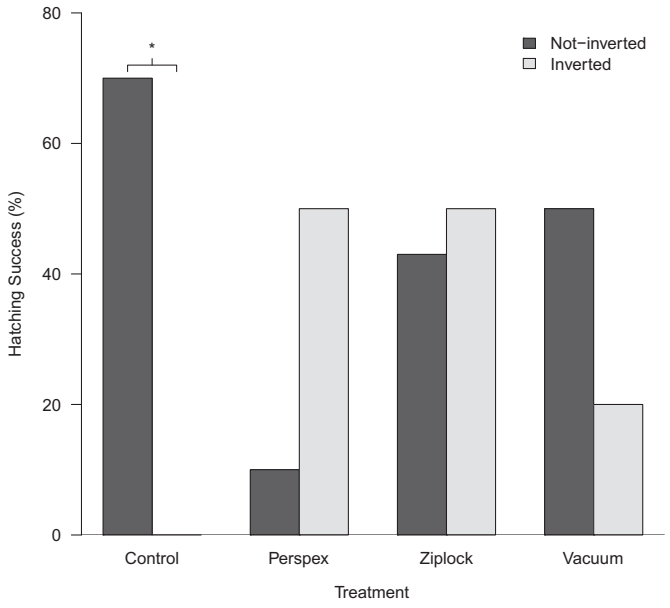


Fig. 4. Hatching success for olive ridley eggs subjected to various treatments and incubated in the laboratory till hatching. Treatments were either control (normoxia; $N = 20$), Perspex chambers ($N = 20$) or ziplock bags ($N = 15$) into which nitrogen gas was pumped to exclude oxygen, or vacuum-sealed 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 ($p \leq 0.05$).

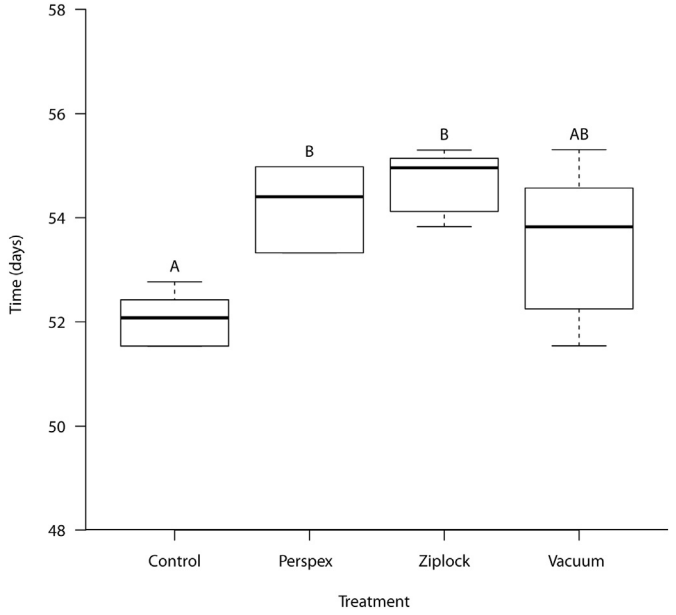


Fig. 5. Incubation length of olive ridley eggs placed into four treatments immediately after oviposition. The four treatments lasted three days and consisted of; 1) a control treatment ($n = 7$), 2) Perspex chambers ($n = 6$) or 3) ziplock bags ($n = 7$) into which nitrogen gas was pumped to exclude 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.

Acknowledgements

S.W. was financially supported throughout his PhD with an Australian Postgraduate Award. This project was supported by the Holsworth Wildlife Research Endowment – Equity Trustees Charitable Foundation. We are grateful for the assistance provided by J. Black, B. Butler, L. Cruz, L. Domico, T. Dornfeld, L. Hackney, C. Hertz, L. McKenna, F. Paladino, G. Shillinger, E. Solano and J. Spotila at the Leatherback Trust and the Goldring-Gund Marine Biology Station. We thank the numerous Earthwatch Institute volunteers for their assistance in the field and laboratory. We appreciate the support of R. Zúñiga-Muños and the Area de Conservación Tempisque for our research. We thank P. Santidrián-Tomillo for assistance with permit applications. Y. Arguello-Gómez and C. Orrego helped with coordinating logistics in the field at Playa Ostional. We are very appreciative and thankful for the support and use of facilities offered by R. Piedra and the staff at Parque Nacional Marino Las Baulas. Finally thanks to C. Neilson for invaluable assistance, inspiration and support in the field and laboratory.

Appendix A. Supplementary data

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

References

Blanck, C.E., Sawyer, R.H., 1981. Hatchery practices in relation to early embryology of the loggerhead sea turtle, *Caretta caretta* (Linné). J. Exp. Mar. Biol. Ecol. 49, 163–177.
Booth, D.T., 2000. Incubation of eggs of the Australian broad-shelled turtle, *Chelodina expansa* (*Testudinata: Chelidae*), at different temperatures: effects on pattern of oxygen consumption and hatchling morphology. Aust. J. Zool. 48, 369–378.
Booth, D.T., 2002. The breaking of diapause in embryonic broad-shell river turtles (*Chelodina expansa*). J. Herpetol. 36, 304–307.
Booth, D.T., Feeney, R., Shibata, Y., 2013. Nest and maternal origin can influence morphology and locomotor performance of hatchling green turtles (*Chelonia mydas*) incubated in field nests. Mar. Biol. 160, 127–137.
Caillouet, C.W., Shaver, D.J., Landry, A.M., 2015. Kemp's ridley sea turtle (*Lepidochelys kempii*) head-start and reintroduction to Padre Island National Seashore, Texas. Herpetol. Conserv. Biol. 10, 309–377.

Chan, E.-H., 1989. White spot development, incubation and hatching success of leatherback turtle (*Dermochelys coriacea*) eggs from Rantau Abang, Malaysia. Copeia 1989, 42–47.
Chan, E.-H., Salleh, H.U., Liew, H.-C., 1985. Effects of handling on hatchability of eggs of the leatherback turtle, *Dermochelys coriacea*. Pertanika 8, 265–271.
Crastz, F., 1982. Embryological stages of the marine turtle *Lepidochelys olivacea*. Rev. Biol. Trop. 30, 113–120.
Deeming, C.D., 1991. Reasons for the dichotomy in egg turning in birds and reptiles. In: Deeming, C.D., Ferguson, M.W. (Eds.), Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles. Cambridge University Press, Cambridge, UK, pp. 307–324.
Dirzo, R., Young, H.S., Galetti, M., Ceballos, G., Isaac, N.J.B., Collen, B., 2014. Defaunation in the Anthropocene. Science 345, 401–406.
Dodd, C.K., Seigel, R.A., 1991. Relocation, repatriation, and translocation of reptiles and amphibians: are they conservation strategies that work? Herpetologica 47, 336–350.
Eckert, K.L., Eckert, S.A., 1990. Embryo mortality and hatch success in in situ and translocated leatherback sea turtle *Dermochelys coriacea* eggs. Biol. Conserv. 53, 37–46.
Ewert, M.A., 1985. Embryology of turtles. In: Gans, C., Billett, F., Manderson, P.F.A. (Eds.), Biology of the Reptilia. John Wiley & Sons, New York, pp. 75–267.
Ewert, M.A., 1991. Cold torpor, diapause, delayed hatching and aestivation in reptiles and birds. In: Deeming, D.C., Ferguson, M.W.J. (Eds.), Egg Incubation: Ifs Effects on Embryonic Development in Birds and Reptiles. Cambridge University Press, Cambridge, UK, pp. 173–191.
Ewert, M.A., Wilson, D.S., 1996. Seasonal variation of embryonic diapause in the striped mud turtle (*Kinosternon baurii*) and general considerations for conservation planning. Chelonian Conserv. Biol. 2, 43–54.
Ferguson, M.W.J., 1985. Reproductive biology and embryology of the crocodilians. In: Gans, C., Billett, F., Maderson, P.F.A. (Eds.), Biology of the Reptilia. John Wiley & Sons, New York, pp. 329–491.
Fordham, D., Georges, A., Corey, B., 2006. Compensation for inundation-induced embryonic diapause in a freshwater turtle: achieving predictability in the face of environmental stochasticity. Funct. Ecol. 20, 670–677.
Fordham, D.A., Georges, A., Corey, B., 2007. Optimal conditions for egg storage, incubation and post-hatching growth for the freshwater turtle, *Chelodina rugosa*: science in support of an indigenous enterprise. Aquaculture 270, 105–114.
Fuentes, M.M.P.B., Fish, M.R., Maynard, J.A., 2012. Management strategies to mitigate the impacts of climate change on sea turtle's terrestrial reproductive phase. Mitig. Adapt. Strateg. Glob. Chang. 17, 51–63.
Garcia, A., Ceballos, G., Adaya, R., 2003. Intensive beach management as an improved sea turtle conservation strategy in Mexico. Biol. Conserv. 111, 253–261.
Germano, J.M., Bishop, P.J., 2009. Suitability of amphibians and reptiles for translocation. Conserv. Biol. 23, 7–15.
Hamann, M., et al., 2010. Global research priorities for sea turtles: informing management and conservation in the 21st century. Endanger. Species Res. 11, 245–269.
Harry, J., Limpus, C., 1989. Low-temperature protection of marine turtle eggs during long-distance relocation. Wildl. Res. 16, 317–320.
Hoegh-Guldberg, O., Hughes, L., McIntyre, S., Lindenmayer, D.B., Parmesan, C., Possingham, H.P., Thomas, C.D., 2008. Assisted colonization and rapid climate change. Science 321, 345–346.
Kennett, R., Georges, A., Palmerallen, M., 1993. Early developmental arrest during immersion of eggs of a tropical fresh-water turtle, *Chelodina rugosa* (*Testudinata*, *Chelidae*), from northern Australia. Aust. J. Zool. 41, 37–45.
Kornaraki, E., Matossian, D.A., Mazaris, A.D., Matsinos, Y.G., Margaritoulis, D., 2006. Effectiveness of different conservation measures for loggerhead sea turtle (*Caretta caretta*) nests at Zakynthos Island, Greece. Biol. Conserv. 130, 324–330.
Leslie, A.J., Penick, D.N., Spotila, J.R., Paladino, F.V., 1996. Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990–1991. Chelonian Conserv. Biol. 2, 159–168.
Limpus, C.J., Baker, V., Miller, J.D., 1979. Movement induced mortality of loggerhead eggs. Herpetologica 35, 335–338.
McDonald-Madden, E., Runge, M.C., Possingham, H.P., Martin, T.G., 2011. Optimal timing for managed relocation of species faced with climate change. Nat. Clim. Chang. 1, 261–265.
Miller, J.D., 1985. Embryology of marine turtles. In: Gans, C., Billett, F., Maderson, P.F.A. (Eds.), Biology of the Reptilia. John Wiley & Sons, New York, pp. 269–328.
Miller, J.D., 1999. Determining clutch size and hatching success. In: Eckert, K.L., Bjorndal, K.A., Abreu-Grobois, F.A., Donnelly, M. (Eds.), Research and Management Techniques for the Conseravtion of Sea Turtles. IUCN/SSC Marine Turtle Specialist Group Publication No. 4pp. 124–129.
Miller, J.D., Limpus, C.J., 1983. A method for reducing movement-induced mortality in turtle eggs. Mar. Turt. Newsl. 10–11.
Mitchell, N.J., Rodriguez, N., Kuchling, G., Arnall, S.G., Kearney, M.R., 2016. Reptile embryos and climate change: modelling limits of viability to inform translocation decisions. Biol. Conserv. 204, 134–147.
Parmenter, C.J., 1980. Incubation of the eggs of the green sea turtle, *Chelonia mydas*, in Torres Strait, Australia - the effect of movement on hatchability. Aust. Wildl. Res. 7, 487–491.
Pfaller, J.B., Limpus, C.J., Bjorndal, K.A., 2009. Nest-site selection in individual loggerhead turtles and consequences for doomed-egg relocation. Conserv. Biol. 23, 72–80.
Pike, D.A., Roznik, E.A., Bell, I., 2015. Nest inundation from sea-level rise threatens sea turtle population viability. R. Soc. Open Sci. 2, 150127.
Pintus, K.J., Godley, B.J., McGowan, A., Broderick, A.C., 2009. Impact of clutch relocation on green turtle offspring. J. Wildl. Manag. 73, 1151–1157.
R Core Team, 2013. R: A language and environment for statistical computing. In: R Foundation for Statistical Computing, . Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.
Rafferty, A.R., Reina, R.D., 2012. Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles. Proc. R. Soc. B 279, 2299–2308.
Rafferty, A.R., Reina, R.D., 2014. The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. J. Exp. Mar. Biol. Ecol. 450, 91–97.
Rafferty, A.R., Santidrián Tomillo, P., Spotila, J.R., Paladino, F.V., Reina, R.D., 2011. Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*). PLoS One 6, e21038.
Rafferty, A.R., Evans, R.G., Scheelings, F.T., Reina, R.D., 2013. Limited oxygen availability in utero may constrain the evolution of live birth in reptiles. Am. Nat. 181, 245–253.
Rees, A.F., et al., 2016. Are we working towards global research priorities for management and conservation of sea turtles? Endanger. Species Res. 31, 337–382.
Rings, C.C., Rafferty, A.R., Guinea, M.L., Reina, R.D., 2015. The impact of extended preovipositional arrest on embryonic development and hatchling fitness in the flatback sea turtle. Physiol. Biochem. Zool. 88, 116–127.
Safina, C., 2011. The 2010 Gulf of Mexico oil well blowout: a little hindsight. PLoS Biol. 9, e1001049.
Seddon, P.J., Griffiths, C.J., Soorae, P.S., Armstrong, D.P., 2014. Reversing defaunation: restoring species in a changing world. Science 345, 406–412.
Shaver, D.J., Lamont, M.M., Maxwell, S., Walker, J.S., Dillingham, T., 2016. Head-started Kemp's ridley turtle (*Lepidochelys kempii*) nest recorded in Florida: possible implications. Chelonian Conserv. Biol. 15, 138–143.
Sieg, A.E., Binckley, C.A., Wallace, B.P., Tomillo, P.S., Reina, R.D., Paladino, F.V., Spotila, J.R., 2011. Sex ratios of leatherback turtles: hatchery translocation decreases metabolic heating and female bias. Endanger. Species Res. 15, 195–204.
Thompson, M.B., 1985. Functional significance of the opaque white patch in eggs of *Emydura macquarii*. In: Grigg, G., Shine, R., Ehmann, H. (Eds.), Biology of Australasian Frogs and Reptiles. Surrey Beatty in association with The Royal Zoological Society of New South Wales, Chipping Norton, NSW, pp. 387–395.
Tuttle, J., Rostal, D., 2010. Effects of nest relocation on nest temperature and embryonic development of loggerhead sea turtles (*Caretta caretta*). Chelonian Conserv. Biol. 9, 1–7.
Valverde, R.A., Wingard, S., Gómez, F., Tordior, M.T., Orrego, C.M., 2010. Field lethal incubation temperature of olive ridley sea turtle *Lepidochelys olivacea* embryos at a mass nesting rookery. Endanger. Species Res. 12, 77–86.
Williamson, S.A., Evans, R.G., Reina, R.D., 2017. When is embryonic arrest broken in turtle eggs? Physiol. Biochem. Zool. 89, 523–532.
Wyneken, J., Burke, T.J., Salmon, M., Pedersen, D.K., 1988. Egg failure in natural and relocated sea turtle nests. J. Herpetol. 22, 88–96.

Appendix C. Scheelings et al. (2016) *Journal of Wildlife Diseases publication* PDF

Photo used with permission from T. Franciscus Scheelings.

DOI: 10.7589/2015-03-064

Journal of Wildlife Diseases, 52(4), 2016, pp. 959–961
© Wildlife Disease Association 2016

Hematology and Serum Biochemistry for Free-ranging Freshwater Crocodiles (*Crocodylus johnstoni*) in Western Australia

T. Franciscus Scheelings,[1,2] Sean A. Williamson,[1] and Richard D. Reina[1] [1]School of Biological Sciences, Monash University, Clayton VIC 3800, Australia; [2]Corresponding author (email: fscheelings@zoo.org.au)

ABSTRACT: We determined reference ranges for hematology and serum biochemistry in 39 free-ranging freshwater crocodiles (*Crocodylus johnstoni*). Animals were captured from Windjana Gorge in Western Australia and physically restrained for blood sampling from the supravertebral vein. No significant sex differences were found. Four animals were infected with a haemogregarine-like intraerythrocytic parasite.

The freshwater crocodile (*Crocodylus johnstoni*) is a medium-sized member of the family Crocodylidae and is one of two extant species of crocodilians found in Australia. They are distributed along the coast and hinterland of northern Australia, with their range extending from Queensland to Western Australia (Cogger 2014). Freshwater crocodiles are common in captivity in Australia; however, little has been reported on normal physiologic data for these animals. Therefore, health assessment of both captive and free-ranging crocodiles is potentially hampered. We estimated hematology and serum biochemistry reference values from free-ranging, physically restrained freshwater crocodiles in Western Australia.

This study was conducted in the Windjana Gorge National Park, Western Australia (17°24′32″S, 124°57′28″E) from 3–5 September (dry season) 2014. Crocodiles were captured from pools using drift nets or noose poles and brought onto shore. They were then restrained by tying their mouths closed with rope and securing their hind limbs to the tail base with rope and duct tape. Sex was determined by digital cloacal palpation and extrusion of the penis or clitoris. We captured 29 males and 10 females. Basic morphometric data including head length, head width, neck width, snout–vent length, total length, and weight were obtained from most animals (20 males and seven females; Table 1). Blood was collected from the supravertebral vein using a 5-mL syringe connected to an 18-ga needle within 30 min of capture. An equal volume of blood was immediately transferred into a lithium heparin container (BD Microtainer™ Tubes, Becton Dickinson, Franklin Lakes, New Jersey, USA) and a plain BD Microtainer Tube (Becton Dickinson). The packed cell volume (PCV) was determined using standard centrifugation in microhematocrit tubes. In the field at collection, a drop of fresh blood (without anticoagulant) was placed onto a microscope slide, smeared using the bevelled edge of another slide, then air-dried and stained with Romanowsky stain (Rapid Diff, Australian Biostain Pty. Ltd., Traralgon, Victoria, Australia). Leukocyte differential counts were performed manually on blood films, and white cells were classified as heterophils, lymphocytes, eosinophils, basophils, or monocytes (Campbell and Ellis 2007). Heterophil/eosinophil counts were performed manually using a hemocytometer and by staining whole blood with phloxine B (made in-house). The total white blood cell (TWBC) count was calculated by correcting the manual count for the percentage of heterophils and eosinophils present (Dein et al. 1994). Both PCV and TWBC counts were determined within 24 h of blood collection. Blood in the plain tube was chilled in a portable ice pack (–20 C) in the field and centrifuged back at camp. The resulting serum was transported to the lab on dry ice and frozen at –70 C for up to 5 mo until analysis. Serum was analyzed using the avian-reptilian rotor on the Vet Scan analyzer (Abaxis, Union City, California, USA). Parameters included aspartate aminotransferase, creatine kinase, total protein, uric acid, glucose, calcium, phosphate, albumin, globulin, potassium, and sodium (Table 2). Once experimentation was completed all crocodiles were returned to their exact point of capture.

TABLE 1. Morphometric data for 20 male and seven female free-ranging freshwater crocodiles (*Crocodylus johnstoni*) captured at Windjana Gorge in Western Australia, September 2014.

Measurement	Male (mean±SE)	Female (mean±SE)
Head length (cm)	27.8±2.6	25.8±1.6
Head width (cm)	12±1.6	11.6±1.4
Neck width (cm)	37.4±4.0	34.4±3.7
Snout–vent length (cm)	86.7±9.3	79.8±4.4
Total length (cm)	169.9±20.5	152.3±7.9
Weight (kg)	14.8±9.1	8.7±3.2

All procedures were approved by the Monash University School of Biological Sciences Ethics Committee (BSCI/2013/23), Clayton, Victoria, Australia. Collection of crocodiles was approved with permits obtained from the Western Australian Department of Parks and Wildlife (Permit SF009976).

Data were imported into Microsoft Excel 2007 (Microsoft Corporation, Redmond, Washington, USA) and statistical analysis was performed using SYSTAT (Systat Software Inc., Chicago, Illinois, USA). Parametric and nonparametric methods were used to determine reference values with 95% confidence intervals (American Society for Veterinary Clinical Pathology 2011). For all data collected, ranges were calculated by Dixon Q test analysis of data with outliers (defined by a D/R ratio greater than 1:3, where D=the difference between the most extreme values and R=the range of values) excluded. Data were assessed for normality with the Shapiro-Wilk test and analyses of variance were conducted to identify whether there was a significant difference in hematologic and biochemical values between sexes. Linear mixed models using the "LME" function in the 'nlme' package of R (R Development Core Team 2015) were used to determine if crocodile body morphometrics were predicted by hematologic and biochemical values. Significance was accepted at $P \leq 0.05$.

TABLE 2. Hematology and serum biochemistry results for free-ranging freshwater crocodiles (*Crocodylus johnstoni*) captured at Windjana Gorge in Western Australia, September 2014. Ranges are compared against reference intervals for the Nile crocodile (*Crocodylus niloticus*; Lovely et al. 2007), saltwater crocodile (*Crocodylus porosus*; Milan et al. 1997), and the mugger crocodile (*Crocodylus palustris*; Stacey and Whitaker 2000).[a]

Parameter	Range	Mean±SE	*n*	Nile crocodile (min–max)	Saltwater crocodile	Mugger crocodile
PCV (%)	18.0–32.0	23.1±3.4	39	14.0–22.0	17.0–41.0	16.0–38.0
TWBC (10^3/μL)	2.2–17.5	8.0±3.9	39	3.7–26.2	6.4–25.7	5.1–15.4
Heterophils (10^3/μL)	0.1–6.8	1.3±1.3	39	0.4–3.7	0.8–7.4	3.1–9.5
Lymphocytes (10^3/μL)	1.4–9.8	5.1±2.5	39	1.6–17.8	4.5–21.6	1.2–4.9
Monocytes (10^3/μL)	0.0–2.7	1.0±0.8	39	0.0–0.8	0.0–1.2	0.0–0.3
Eosinophils (10^3/μL)	0.0–1.6	0.7±0.5	39	0.0–2.1	0.0–0.7	0.0–1.3
Basophils (10^3/μL)	0.0–0.6	0.0±0.1	39	0.0–2.9	0.0–0.4	0.0–0.1
AST (U/L)	19.0–74.0	36.5±12.8	39	14.0–211.0	23.0–157.0	36.0–70.0
Creatine kinase (U/L)	109.0–4,448.0	1,086.4±1,243	39	NA	NA	7.0–10.0
Uric acid (μmol/L)	27.0–113.0	69.0±25.2	39	40.0–300.0	0.17–0.99	142.7–588.8
Glucose (mmol/L)	2.0–6.2	3.6±1.0	39	1.8–4.8	4.5–12.1	2.7–5.4
Calcium (mmol/L)	1.0–2.9	2.3±0.5	39	1.08–1.61	2.4–3.4	2.9–3.4
Phosphorus (mmol/L)	0.5–1.9	1.2±0.4	39	NA	1.2–2.9	1.3–3.1
Total protein (g/L)	15.0–70.0	39.6±14.9	39	28.9–57.1	41.0–70.0	20.0–41.0
Albumin (g/L)	0–15	7.7±3.9	39	11.1–19.4	14.0–23.0	10.0–14.0
Globulin (g/L)	0–55	15.8±20.7	39	16.5–42.6	27.0–50.0	16.0–28.0
Potassium (mmol/L)	2.3–5.2	3.7±0.8	39	3.3–7.65	3.8–7.2	7.3–8.9
Sodium (mmol/L)	111–154	138.5±9.7	39	122–164	143.0–161.0	133.0–166.0

[a] PCV = packed cell volume; TWBC = total white blood cell; AST = aspartate aminotransferase; NA = values not available.

We found no differences between sexes for any analyte, so data were combined (Table 2). For morphometric data the only significant difference was in total length, with males being longer than females (P=0.039). There were no statistically significant correlations between morphometric data and hematology or serum biochemistry for either sex. We used the supravertebral vein because of the size of the crocodiles and the restraint method. Although there is the risk of sampling the spinal venous sinus using this method (Myburgh et al. 2014), this can be avoided by careful technique. To our knowledge, these are the first hematology and serum biochemistry reference intervals for free-ranging, physically restrained freshwater crocodiles.

We found four male crocodiles infected with a haemogregarine-like intraerythrocytic parasite. Infection prevalence was very low in each of the individuals with less than one in 1,000 red blood cells infected. Infection did not result in statistically significant differences in hematology or biochemistry in these animals. No hemoparasites were observed in any female sample. To our knowledge, this is the first report of hemoparasitism in any species of crocodile in Australia. Further investigation is warranted to identify the species of hemoparasite and to elucidate if there are any physiologic effects to affected individuals.

We thank Nicolette Newell from REM Systems Australia for kindly donating the Abaxis rotors used in this study. Also, many thanks to Ruchira Somaweera from Biologic Environmental Survey as well as Tracey Sonneman and all the volunteers from the Western Australian Department of Parks and Wildlife for the invitation to join them in the Kimberley and for all the help that they provided. Finally we thank the Holsworth Wildlife Research Endowment for financial support.

LITERATURE CITED

American Society for Veterinary Clinical Pathology. 2011. *Guidelines for the determination of reference intervals (RI) in veterinary species*. http://www.asvcp.org/pubs/pdf/RI%20Guidelines%20For%20ASVCP%20website.pdf. Accessed January 2014.

Campbell TW, Ellis CK. 2007. Hematology of reptiles. In: Campbell TW, Ellis CK, editors. *Avian and exotic animal hematology and cytology*. 3rd Ed. Blackwell Publishing, Ames, Iowa, pp. 3–82.

Cogger H. 2014. *Reptiles and amphibians of Australia*. 7th Ed. CSIRO Publishing, Collingwood, Victoria, Australia, 1064 pp.

Dien FJ, Wilson A, Fischer D, Langenberg P. 1994. Avian leucocyte counting using the hemocytometer. *J Zoo Wildl Med* 25:432–437.

Lovely CJ, Pittman JM, Leslie AJ. 2007. Normal haematology and blood biochemistry of wild Nile crocodiles (*Crocodylus niloticus*) in the Okavango Delta, Botswana. *J S Afr Vet Assoc* 78:137–144.

Millan JM, Janmaat A, Richardson C, Chambers LK, Fomiatti KR. 1997. Reference ranges for biochemical and haematological values in farmed saltwater crocodile (*Crocodylus porosus*) yearlings. *Aust Vet J* 75:814–817.

Myburgh JG, Kirberger RM, Steyl JCA, Soley JT, Booyse DG, Huchzermeyer FW, Lowers RH, Guillette LJ Jr. 2014. The post-occipital spinal venous sinus of the Nile crocodile (*Crocodylus niloticus*): Its anatomy and use for blood sample collection and intravenous infusions. *J S Afr Vet Assoc* 85:965.

Stacy BA, Whitaker N. 2000. Hematology and blood biochemistry of captive mugger crocodiles (*Crocodylus palustris*). *J Zoo Wildl Med* 31:339–347.

R Development Core Team. 2015. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-Project.org/. Accessed May 2016.

Submitted for publication 18 March 2015.
Accepted 7 July 2015.

Appendix D. Robinson *et al.* (2016) *Marine Turtle Newsletter publication* PDF

Plastic Fork Found Inside the Nostril of an Olive Ridley Sea Turtle

Nathan J. Robinson[1,2], Tera C. Dornfeld[2,3], Brett O. Butler[2], Lia J. Domico[2], Collin R. Hertz[2], Lindsay N. McKenna[1,2], Chenae B. Neilson[2] & Sean A. Williamson[2,4]

[1]Department of Biology, Indiana University-Purdue University Fort Wayne, Fort Wayne, IN, USA (E-mail: [redacted]

[2]The Leatherback Trust, Goldring-Gund Marine Biology Station, Playa Grande, Guanacaste, Costa Rica (E-mail: [redacted]

[3]School of Social Ecology, University of California Irvine, Irvine, CA, USA [redacted]

[4]School of Biological Sciences, Monash University, Melbourne, Victoria, Australia [redacted]

The accumulation of plastic debris in the world's oceans poses a growing threat to marine life that they may incidentally ingest or become entangled by (Jambeck *et al.* 2015; Rochman *et al.* 2016). Furthermore, as plastic debris continues to accumulate in the oceans there will likely be a concurrent increase in the frequency and diversity of interactions between marine life and plastic debris. Recently, we described the first-ever incidence of a plastic straw in the nostril of an olive ridley sea turtle *Lepidochelys olivacea* (Robinson & Figgener 2015). Here, we describe a similar event; the discovery of a plastic fork in the nostril of an olive ridley sea turtle.

We encountered the olive ridley turtle at Ostional beach on the Pacific coast of Costa Rica (9° 56', 85° 39', Fig. 1). Playa Ostional hosts sea turtle mass-nesting events, termed *arribadas*. We were present during one such *arribada* on 6 December 2015 to collect epibionts from nesting olive ridley turtles. While conducting our research, we were alerted about a nesting olive ridley turtle that had a foreign body protruding approximately 4 cm from its left nostril (Fig. 2a) that was likely impairing the turtle's ability to breathe. Having immediate concerns for the turtle's health, we therefore attempted to gently manipulate the foreign body.

The foreign body appeared firmly lodged in place; however, bringing the turtle in for veterinary treatment was not an option. It would take many hours to drive to the nearest veterinary clinic and we had no assurance that appropriate treatment would be available. In addition, moving the animal would have been in violation of our permits, which only allowed us to restrain the turtle for a maximum of 20 min – the time required to exhaustively collect epibionts from a nesting sea turtle. Thus, we decided to remove the fork *in situ*.

We firmly gripped on the protruding end of the foreign body with a Swiss Army knife. After only a short pull, the object came free from the turtle's nose (Fig. 2b). Upon removal, we discovered that the foreign body was a plastic fork. The fork measured 13 cm long (Fig. 2c), and approximately 9 cm of the fork, including the tines, had been inside the sea turtle's nasal passage before removal. Shortly after the fork was removed, the turtle appeared healthy and active and crawled back to the ocean and swam away.

This is the second report of a plastic foreign body in the nostril of a sea turtle, the first being a plastic straw (Robinson & Figgener 2015). Interestingly, the straw was discovered nearby in the waters in front of Playas Del Coco only 5 months previous (Fig. 1; approx. 60 km north of Playa Ostional) and it also affected an olive ridley turtle. Two such events in close proximity and in a short time frame could be attributable to increased surveillance effort. Indeed, both the straw and the fork were discovered as part of the same study on epibiont diversity that began in 2014 and requires us to closely inspect sea turtles for epibionts. However, scientists have been monitoring nesting olive ridley sea turtles along the Pacific coast of Costa Rica for many years (e.g., Fonseca *et al.* 2009; Valverde *et al.* 2012; Dornfeld *et al.* 2015) and such an event had not previously been recorded. Thus, increased surveillance alone is unlikely to explain these recent discoveries. Instead, we consider that these recent discoveries are the product of accumulating plastic debris in the waters of northwest Costa Rica. Increased quantities of plastic debris could be attributed to either the steady increase in plastic pollution from local sources (e.g., riverine input) or dynamic oceanographic processes that may lead to local aggregations of plastic debris from potentially distant sources (Duhec *et al.* 2015).

The direction that the fork was pointing may provide some crucial insights as to how the fork (and perhaps straw) came to be found within turtles' noses. As the fork was found with the tines pointed posteriorly into the turtle's nasal passage, it is highly unlikely that the fork could have entered the external nares by force. Instead, we hypothesize that the turtle first ingested the plastic fork and then tried to regurgitate it. Sea turtles regularly ingest plastic objects (Schuyler *et al.* 2014; Nelms *et al.* 2015; Wedemeyer-Strombel *et al.* 2015), presumably after mistaking them for prey items (Schuyler *et al.* 2012). A plastic fork could have been mistaken for a penaeid

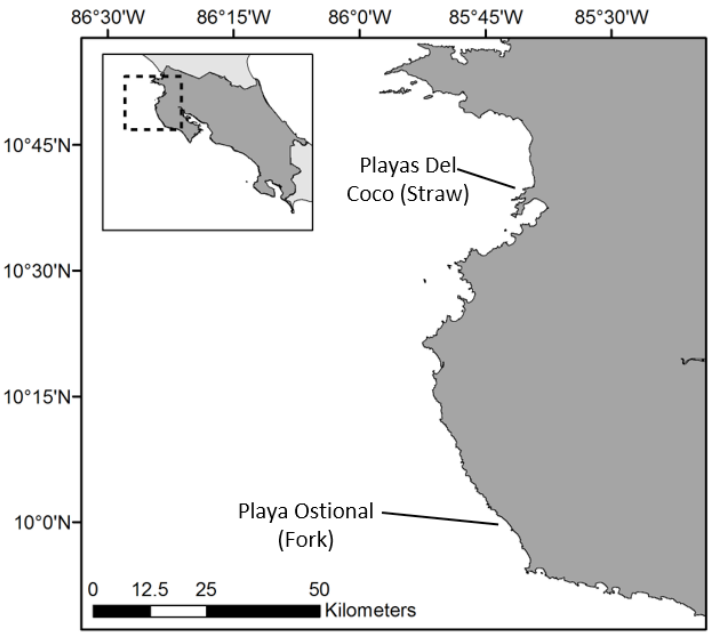


Figure 1. Map of the Pacific Northwest of Costa Rica including the location where both the fork (this study) and the straw (Robinson & Figgener 2015) were removed from the nostril of an olive ridley turtle.

Figure 2. (A) Plastic fork in the right nostril of an olive ridley sea turtle. (B) Removal of the fork. (C) The fork next to a ruler for scale.

shrimp or a crab's appendage - both prey items that are regularly found in the stomachs of olive ridley turtles (Colman *et al.* 2014). Thus, we believe that the turtle initially ingested the fork but then attempted to regurgitate it. Being rigid, the fork was angled toward the roof of the mouth as it was regurgitated. Instead of passing out of the mouth, the fork was consequently passed into the internal nares, which are found on the roof of the mouth (Fig. 3, Wyneken 2001). Moreover, the width of the fork's head stopped the fork from being passing completely out of the nasal passage. This pattern of ingestion and regurgitation is probably the most parsimonious explanation for how a straw also came to be lodged in a sea turtle's nostril (Robinson & Figgener 2015).

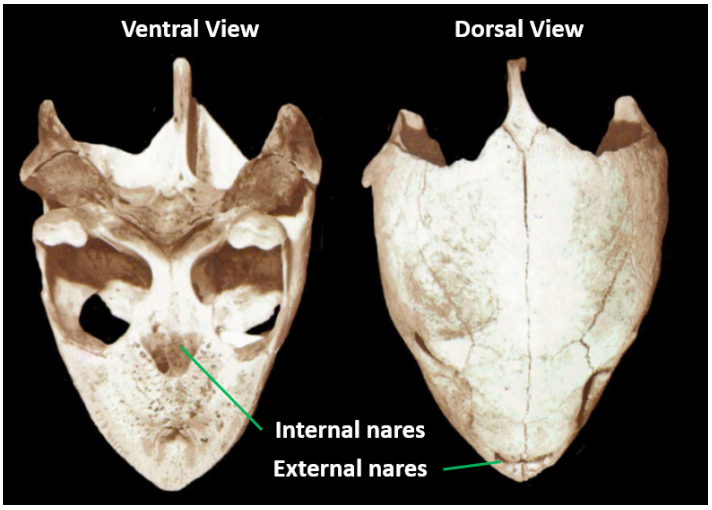


Figure 3. Dorsal and ventral perspectives of an olive ridley sea turtle's skull indicating the position of the external and internal nares. Modified from Wyneken 2001.

While it is often assumed that sea turtles are unable to regurgitate items due to the presence of esophageal papillae (Magalhães *et al.* 2012, Schuyler *et al.* 2014), this is not entirely correct. Sea turtles have regularly been observed to regurgitate partially liquefied foods after being tube fed or receiving a gastric-esophageal lavage (Mendonça 1983; Norton 2005). As turtles can therefore exhibit a regurgitation response, it might therefore be possible for turtles to occasionally regurgitate solid items, such as a plastic fork or straw. This might be especially true if the solid item has only passed as far as the pharynx and not the esophagus. If this is true, and we also consider the quantity of plastic that might pass naturally through the alimentary canal, the quantity of plastic debris found in the stomach of sea turtles is probably an underestimation of the total plastic that these animals ingest over their lives.

Considering that some studies have found plastic in over 80% of all turtles sampled (Schuyler *et al.* 2014; Wedemeyer-Strombel *et al.* 2015), the vast majority of turtles are likely to have had some form of interaction with plastic debris over their life time. Although the full extent of the threat that pollution poses to sea turtles, or most marine life, is still largely unknown (Rochman *et al.* 2016), the overwhelming prevalence of plastic debris in the guts of marine animals is a worrying sign. We predict that over time, methods for reducing plastic pollution on a global scale are likely to play an increasingly necessary role in conservation management strategies for many endangered marine species.

Acknowledgements. This research was conducted under permits from SINAC (#ACT-OR-DR-099-15). We thank Roberto Zúñiga Muños and the Area de Conservación Tempisque for their support of research. We thank Victoronix for providing essential equipment for this study. Pilar Santidrián Tomillo provided assistance with permitting. Yeudy José Arguello Gómez helped coordinate logistics at Playa Ostional.

COLMAN, L.P., C.L.S. SAMPAIO, M.I. WEBER & J.C de CASTILHOS. 2014. Diet of olive ridley sea turtles, *Lepidochelys olivacea*, in the waters of Sergipe, Brazil. Chelonian Conservation & Biology 2014: 266-271.

DORNFELD, T.C., N.J. ROBINSON, P. SANTIDRIÁN TOMILLO & F.V. PALADINO. 2015. Ecology of solitary nesting olive ridley sea turtles at Playa Grande, Costa Rica. Marine Biology 162: 123-139.

DUHEC, A.V., R.F. JEANNE, N, MAXIMENKO & J. HAFNER. 2015. Composition and potential origin of marine debris stranded in the Western Indian Ocean on remote Alphonse Island, Seychelles. Marine Pollution Bulletin 96: 76-86.

FONSECA, L.G., G.A. MURILLO, L. GUADAMÚZ, R.M. SPÍNOLA & R.A. VALVERDE. 2009. Downward but stable trend in the abundance of arribada olive ridley sea turtles (*Lepidochelys olivacea*) at Nancite Beach, Costa Rica (1971–2007). Chelonian Conservation & Biology 8: 19-27.

JAMBECK, J.R., R. GEYER, C. WILCOX, T.R. SIEGLER, M. PERRYMAN, A. ANDRADY, R. NARAYAN & K.L. LAW. 2015. Plastic waste inputs from land into the ocean. Science 347: 768-771.

MAGALHÃES, M.D.S., A.J.B. SANTOS, N.B.D. SILVA & C.E.B.D. MOURA. 2012. Anatomy of the digestive tube of sea turtles (Reptilia: Testudines). Zoologia (Curitiba) 29: 70-76.

MENDONÇA, M.T. 1983. Movements and feeding ecology of immature green turtles (*Chelonia mydas*) in a Florida lagoon. Copeia 1983: 1013-1023.

NELMS, S.E., E.M. DUNCAN, A.C. BRODERICK, T.S. GALLOWAY, M.H. GODFREY, M. HAMANN, P.K. LINDEQUE & B.J. GODLEY. 2015. Plastic and marine turtles: a review and call for research. ICES Journal of Marine Sciences 73: 165-181.

Norton, T. M. 2005. Chelonian emergency and critical care. Seminars in Avian and Exotic Pet Medicine 14: 106-130.

ROBINSON, N.J. & C. FIGGENER. 2015. Plastic straw found inside the nostril of an olive ridley sea turtle. Marine Turtle Newsletter 147: 5-6.

ROCHMAN, C.M., M.A. BROWNE, A.J. UNDERWOOD, J.A. VAN FRANEKER, R.C. THOMPSON & L.A. AMARAL-ZETTLER. 2016. The ecological impacts of marine debris: unraveling the demonstrated evidence from what is perceived. Ecology 97: 302-312.

SCHUYLER, Q., B.D. HARDESTY, C. WILCOX & K. TOWNSEND. 2012. To eat or not to eat? Debris selectivity by marine turtles. PLoS ONE 7: e40884.

SCHUYLER, Q., B.D. HARDESTY, C. WILCOX & K. TOWNSEND. 2014. Global analysis of anthropogenic debris ingestion by sea turtles. Conservation Biology 28: 129-139.

VALVERDE, R.A., C.M. ORREGO, M.T. TORDOIR, F.M. GÓMEZ, D.S SOLÍS, R.A. HERNÁNDEZ, G.B. GÓMEZ, L.S. BRENES, J.P. BALTODANO, L.G. FONSECA & J.R. SPOTILA. 2012. Olive ridley mass nesting ecology and egg harvest at Ostional Beach, Costa Rica. Chelonian Conservation & Biology 11: 1-11.

WEDEMEYER-STROMBEL, K.R., G.H. BALAZS, J.B. JOHNSON, T.D. PETERSON, M.K. WICKSTEN & P.T. PLOTKIN. 2015. High frequency of occurrence of anthropogenic debris ingestion by sea turtles in the North Pacific Ocean. Marine Biology 162: 2079-2091.

WYNEKEN, J. 2001. The anatomy of sea turtles. U.S. Department of Commerce NOAA Tech Memo NMFS-SEFSC-470, 172 pp.