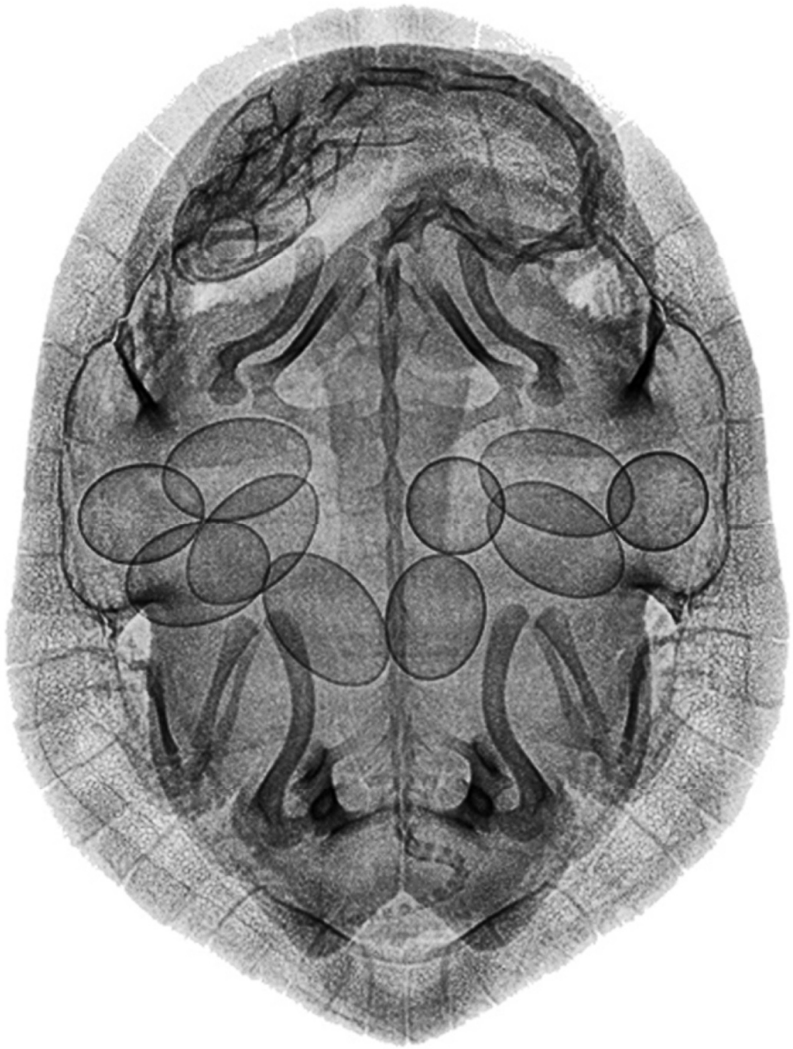
Environmental influences and ecological significance of arrested embryonic development in chelonians



Anthony R. Rafferty

Environmental influences and ecological significance of arrested embryonic development in chelonians

A thesis submitted for the degree of Doctor of Philosophy

Anthony Richard Rafferty

M.E.M., B.Sc.

School of Biological Sciences

Monash University

Melbourne, Australia

June 2012

A juvenile Murray Rive turtle (*Emydura macquarii*)
Photo taken by Anthony. R. Rafferty

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ABSTRACT

Arrested embryonic development is an important reproductive strategy in the large range of egg laying animals that provide no parental care after oviposition, because it gives their eggs some capacity to respond to changing environmental conditions during development. In this study, I used a comparative approach to identify the external environmental stimuli that cause oviparous reptile embryos to arrest development inside the amniotic egg. This was achieved by investigating how development was directed by maternal (pre-ovipositional development) and nest (post-ovipositional) factors including oxygen tension and temperature, respectively, in eggs of the green sea turtle, *Chelonia mydas*, and three species of freshwater turtle; the western oblong turtle, *Chelodina oblonga*, the eastern longneck turtle, *Chelodina longicollis*, and the Murray River turtle, *Emydura macquarii*.

Initially, I conducted an investigation using an existing long-term data set collected during an ongoing beach monitoring project for the leatherback turtle, *Dermochelys coriacea*, at Playa Grande, Costa Rica, to establish whether the duration that embryos remain arrested *in utero* compromises survival after oviposition. This examination revealed that a large proportion of leatherback turtle embryos were failing to resume development after eggs were laid and dying in a state of pre-ovipositional developmental arrest. I then endeavoured to identify the mechanism involved in pre-ovipositional arrest by assessing oviducal O_2 availability and the impact of hypoxia on embryonic development in the 4 mentioned species. Eggs of each species were allocated immediately after oviposition to either a normoxic (155 mmHg O_2) or a hypoxic (~7.6 mmHg) treatment for 3, 6 or 9 days. Embryonic development of all species progressed normally in normoxia, but in hypoxia development remained arrested and recommenced upon subsequent return to normoxic conditions. I also measured PO_2 in oviducts of gravid turtles, with mean oviducal PO_2 of 5.9 ± 2.5 mmHg in *C. oblonga*; 1.6 ± 1.2 mmHg in *C. Longicollis*; 5.3 ± 2.1 mmHg in *E. macquarii*; and 2.9 ± 1.4 mmHg in *C. mydas*. Furthermore, O_2 diffusion was measured in green turtle oviducal secretion samples and was significantly lower in oviducal secretion (1.9 ± 0.6 mmHg min^{-1}) than in saline controls (14.2 ± 2.1 mmHg min^{-1}, $F = 64.3$, $P < 0.01$). These results suggest that the reduced diffusive ability of oviducal secretion contributes to an extremely hypoxic environment in the oviduct that constrains embryonic development in turtles and maintains pre-ovipositional arrest until after eggs are laid.

I also investigated whether incubation temperature influenced arrested embryonic development, by incubating eggs of each species at three different temperatures immediately after oviposition until hatching, and monitoring embryonic development. Results demonstrated that the breaking of pre-ovipositional arrest after eggs are laid occurred independently of temperature in all four species. However, temperature influenced subsequent development beyond this point and at 22ºC, 64% of *E. macquarii* embryos developed to stage 25 of a 26 stage developmental chronology, but failed to hatch. Similarly, at 24ºC, 45% of *C. mydas* embryos failed to hatch and died at stage 30 of a 31 stage developmental chronology. Presumably, this was because embryos of both species entered a state of delayed hatching, awaiting a necessary stimulus to trigger pipping that never arose at constant temperature. These findings represent the first evidence that sea turtles possibly retain remnant traces of an ability to delay hatching that they once possessed, or perhaps that they may be pre-adapted to evolve delayed hatching in the future.

Finally, I examined the trade-offs between reproductive investment and maternal health in each of the freshwater species included in this study and revealed that females of all three species altered their level of reproductive investment based on health state, manifested by changes in egg mass and size, as well as clutch size. These findings agreed with the physiological constraint hypothesis. Furthermore, no trade-offs existed between clutch and egg size in any of the species under investigation, which refuted the optimum egg size hypothesis and agreed with previous findings in other chelonian species.

The findings presented in this thesis contribute to our understanding of how ecology shapes the evolution of developmental processes observed in reptile embryos, in addition to providing evidence of the mechanisms underlying the evolutionary transition between reproductive modes. It sets the foundation for future research, particularly investigations that focus on understanding how maternal effects influence embryonic development both before and after oviposition in reptiles, despite the absence of parental care in many species.

GENERAL DECLARATION

Monash University Research Graduate School

Declaration for thesis based on conjointly published work

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy regulations the following declarations are made:

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 three original papers published in peer reviewed journals and three unpublished manuscripts that are currently under review. The core theme of the thesis is to identify the environmental influences that cause embryonic developmental arrest in chelonians and determine the associated ecological significance. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Dr. Richard Reina.

The inclusion of co-authors reflects the fact that the work came from active collaboration among researchers and acknowledges input into team-based research. In the case of chapters two, three, four, five, six and appendix A, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles	Published	Conception, literature review and manuscript preparation
3	Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*)	Published	Conception, initiation, design, data analysis and manuscript preparation
4	Limited oxygen availability in utero may constrain the evolution of live-birth in reptiles	In review	Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation

5	The influence of temperature on arrested embryonic development in turtles	In review	Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation
6	Maternal health influences reproductive investment in three species of freshwater turtle	In review	Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation
A	Hematologic and biochemical values of gravid freshwater Australian Chelonians	Published	Ethics, permits, data collection and analysis, project coordination and manuscript preparation

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

Signed:

Date:

ACKNOWLEDGEMENTS

It is with great pleasure that I acknowledge all of the turtle folk worldwide, dedicating their time and effort to conserve this fascinating and charismatic group of reptiles. I have thoroughly enjoyed interacting with you all over the last several years and thrived on the lively debates that took place over tequila at the annual ISTS meetings. I am completely in awe of the work that is being done globally and want to recognise that with this thesis.

I would like to extend my warmest thanks to Richard Reina, my project advisor and friend, who has offered guidance and support throughout the years and helped me battle through many project related difficulties. I have enjoyed dropping by your office for a daily rant and appreciate that your door has always been open to me. I would also like to thank Roger Evans and Franciscus Scheelings, who completed this wonderful research team. Working in a collaborative team with such fantastic and enthusiastic people is the reason why I became a scientist, thank you for reminding me of this every time that we are together.

I have had the pleasure of interacting with a multitude of great minds during this project and I am extremely grateful for the time offered by Colin Limpus, Pilar Santidrián Tomillo, James Spotila, Frank Paladino, Bryan Wallace, David Booth, Jeanette Wyneken, Danielle Stockheld, Peter Comber, Gerald Kuchling, Jacqueline Giles, Bob Prince and Gabriela Eppel. The support, co-operation and enthusiasm of Jay Van Rijn and Janine Ferguson also made a large proportion of my field work achievable, which I am tremendously thankful for.

Many thanks also to my Monash colleagues who offered discussion and advice, just a few of whom include Bob Wong, Dave Chapple, Alistair Evans and Paul Sunnucks, as well as Damian Dowling, Andreas Svensson, John Morrongiello and Jodi Neary. Additionally, I would have been lost without my daily interactions and conversations with my office mates Nicole Kowalczyk, Brian Kearney, Leo Guida, Christopher Johnstone and Tiana Preston who have all provided a great source of statistical information, entertainment and procrastination. Thanks also to the other Monash graduate students that brightened up each day including Krystina Mossop, Ben Wagner, Rebecca Bray, Noriyoshi Kawasaki, Giselle Perdomo and Theresa Mackintosh. Belinda Lees and Ricardo San Martin have also been invaluable throughout the PhD process and I would like to thank them both wholeheartedly for their help.

Thank you so much to the many organisations that supported this research including Austvet, Heron Island Research Station and Zoos Victoria, namely those at Melbourne Zoo and the wonderful staff at Healesville Sanctuary Wildlife Health Centre. I am also grateful for the financial support provided by Monash University and the Holsworth Wildlife Fund.

And last, but definitely not least, thank you so much to my partner Bryan for hauling me out of several deep lakes and for always listening to my project related dramas over the years, and my extremely supportive family, Bernice, Tony, Aisling and Béibhinn, who have all taught me to be myself and chase my dreams with vivacity. This thesis is dedicated to all of you.

Chapter One

General introduction

Study species including the oblong turtle (*Chelodina oblong*; top left), eastern long-necked turtle (*Chelodina longicollis*; top right), Murray River turtle (*Emydura macquarii*; bottom left) and green turtle (*Chelonia mydas*; bottom right)
Photos taken by Anthony R. Rafferty (top and bottom left, top right) and T. Franciscus Scheelings (bottom right)

1.1 Brief overview

Evolution of the amniotic egg was paramount in eliminating the need for amniotes to reproduce in water, because it facilitated the evolution of water-dwelling amphibians into terrestrial reptiles and allowed embryos to exist on land without parental care (Reisz 1997). However, amniotic eggs are exposed to a highly variable incubation environment on land in comparison to the relatively stable aquatic system (Kohring 1995). Therefore, in order to deal with potentially adverse conditions, several reptilian lineages subsequently evolved viviparity (live birth), while those that retained oviparity (egg laying) became capable of arresting embryonic development inside the egg until favourable conditions arise or return (Shine 2005).

Arrested embryonic development is the down-regulation or cessation of cell division and metabolic activity (Ewert 1991) and is seen in many taxa (Thom *et al.* 2004), although extant reptiles (squamates, crocodiles, turtles and the tuatara) possess the most diverse range of strategies to prolong the egg state (Ewert 1991). In doing so, they have the capacity to resist environmental conditions that do not favour successful development of the embryo and can synchronise hatching with seasonal periods that benefit the resulting offspring (Renfree & Shaw 2000).

Although the view that developmental arrest occurs in response to environmental factors associated with an embryo's external environment is widely held, very little is known about the triggers and regulators of such an important developmental strategy. Furthermore, a substantial amount of past investigation has ignored the adaptive significance of life history traits such as developmental arrest and how they influence embryonic development and the timing of hatching. This has resulted in a poor understanding of how environment pressures shape the evolution of processes associated with embryonic development and ontogeny (Van Valen 1976; Sultan 2003). Expanding our understanding of these developmental strategies will greatly advance our knowledge of the evolution of amniote reproduction beyond oviparity. Furthermore, it will also assist in uncovering some of the secrets associated with terrestrial invasion and the evolution of incredible ecological adaptations, including transitions in reproductive mode, which have come about in response to challenges associated with laying shelled eggs on land (Bennett 1987).

1.2 Study aims

The aim of this thesis was to identify the external environmental stimuli that cause oviparous reptile embryos to arrest development inside the amniotic egg, allowing us to answer several fundamental questions about the developmental strategies that have evolved to promote embryo survival in the absence of parental care. This was achieved by investigating how development was directed by maternal (pre-ovipositional development) and nest (post-ovipositional) factors including oxygen tension and temperature, respectively. I opted for a comparative approach to this research, including four species of turtle, consisting of both marine and freshwater species from three different genera. Turtles are an ideal study animal for this type of research because they possess an array of strategies to arrest embryonic development that are more diverse than those seen in other reptile groups. Additionally, several studies have characterised the entire process of embryonic development in both marine and freshwater turtle species (Ewert 1985; Miller 1985), facilitating our investigation. Thesis research primarily focused on pre-ovipositional arrest because although it is universal in turtles, it is the least studied form of arrest due to the difficulties associated with identifying how embryonic development progresses inside a female without sacrificing them and their eggs. However, several other types of arrest including cold torpor, embryonic diapause, aestivation and delayed hatching are also investigated and discussed. Specifically, I used a combination of field and laboratory techniques in addition to analysis of a long-term data set to address the following aims:

• Aim 1: Establish whether mothers can influence pre- and post-ovipositional development and success of their embryos, despite the absence of parental care after eggs are laid.
• Aim 2: Characterise the internal environment of the turtle oviduct and determine whether limited oxygen availability *in utero* influences embryonic development prior to oviposition.
• Aim 3: Determine how incubation temperature influences post-ovipositional development and hatching.

1.3 Study species

Chelodina longicollis, the eastern long-necked turtle, ranges from eastern South Australia, throughout Victoria and most of New South Wales and into eastern Queensland (Cogger 1992). *C. longicollis* typically mate in September and nest between October and January, laying up to three clutches annually that can consist of up to 23 eggs per clutch (Kennett *et al.* 2009). Incubation

generally takes between 110 – 150 days with hatchlings emerging in Autumn in most instances, although hatchlings may overwinter in the nest in some locations (Kennett *et al.* 2009). *Chelodina oblonga*, the oblong turtle, is confined to south-west Western Australia and mating takes place during late winter and spring with nesting occurring between September and January. This species generally lay up to three clutches of up to 16 eggs each per annum (Kuchling 1988; Kuchling 1989). Egg incubation takes approximately 210 - 220 days under natural conditions (Clay 1981), although it may be considerably extended, taking up to 291 days (MCCutcheon 1985). However, overwintering in this species may possibly be occurring, perhaps helping to explain why egg incubation and emergence is so protracted in natural nests (Clay 1981).

The short-necked turtle, *Emydura macquarii*, shares a similar distribution pattern to that described for *C. longicollis*, although mating in this species is usually observed between March and April (Cann 1998). Nesting then takes place between October and December, during which females produce a single clutch of up to 35 eggs (Chessman 1986; Spencer 2001). In natural nests, the average incubation period described for this species is 75 days (Goode & Russell 1968). Interestingly, eggs of *E. macquarii* that are deposited at the same time of year, and under the same conditions as *C. longicollis*, have much shorter incubation periods than *C. longicollis*. Reports on embryonic development in both species suggest that at a constant temperature of 30°C, the embryos of both species appear to be at similar stages of development after 40 days (Goode & Russell 1968). However, beyond this stage, differences in the orientation of the embryo within the eggshell may reduce the ability of *C. longicollis* embryos to ingest yolk in comparison to *E. macquarii* embryos, subsequently reducing their rate of development and extending the incubation time (Goode & Russell 1968). Nevertheless, this fails to explain the incubation times for *C. oblonga*, which are almost double that of *C. longicollis*, suggesting other strategies that prolong or delay development are involved.

Finally, *Chelonia mydas*, the green sea turtle, has a circumglobal distribution with nesting occurring in over 80 countries worldwide following significant trans-continental migrations (Hirth 1997). In northern Queensland, Australia, mating is observed between September and November (Limpus 1993), with nesting occurring between October and March (Bustard 1972). On average, females nest every 5 years or so, during which they will typically lay approximately 5-6 clutches every two weeks (Limpus *et al.* 1994). The mean egg incubation period in this region is about 65 days (Limpus 2008), somewhat similar to that observed in *E. macquarii* nests (Goode & Russell 1968).

Although a myriad of species could have been used for this comparative study, these four mentioned turtle species represent a diversity of the large natural variation in reproductive habits and the developmental timing of embryos that occur among and within different turtle genera. Additionally, they were also easier to obtain for practical and logistical reasons. Firstly, the abundance and timing of reproduction of each species meant that a large number of gravid females could be collected within a single reproductive season. Secondly, the existence of research teams already working with each species provided resources to easily collect gravid specimens in different states. Thirdly, consultation with ethics and permitting authorities revealed that approval could be obtained with ease for each of these species. Finally, published literature suggested that the likelihood of successful artificial egg incubation for these groups was high.

1.4 Thesis structure

This thesis consists of seven chapters, including a general introduction and discussion, and an appendix. Chapter one, this general introduction, is brief because chapter two comprises of a published literature review (Rafferty & Reina 2012a) that provides a detailed overview of each type of developmental arrest observed in oviparous reptiles. Chapter three (aim 1) consists of a published manuscript (Rafferty *et al.* 2011) that investigates the impact that mothers can have on both pre- and post-ovipositional development and success of their embryos, despite the absence of parental care after eggs are laid. This was achieved using a long-term nesting dataset for leatherback turtles, collected during several years of beach monitoring in Costa Rica. Chapter four (aims 1 and 2) is a manuscript, currently under review (Rafferty *et al.* 2012a), dedicated to characterising the internal environment of the reptilian oviduct. Specifically, this chapter determines the effect that limited oxygen availability *in utero* has on embryonic development and the role that it plays in pre-ovipositional arrest. It also tells us whether the evolution of viviparity in some reptile lineages is constrained by their need to arrest embryonic development prior to oviposition. Chapter five (aim 3) is also a manuscript currently under review (Rafferty & Reina 2012b) that examines how incubation temperature dictates development between oviposition and hatching, and in particular, the role that it plays in the breaking of pre-ovipositional arrest after eggs are laid. Although chapter six is not directly related to developmental arrest, this manuscript that is currently under review (Rafferty *et al.* 2012b), describes the influence that maternal physiological health state has on reproductive investment in turtles and highlights the trade-offs that exist between these two physiological systems. Similarly, Appendix A, a published manuscript (Scheelings & Rafferty 2012), is also unrelated to developmental arrest but documents

haematological and biochemical references ranges for all three freshwater species included in this study and examines whether body size is a function of physiological health state in reproductive female turtles. Both of these latter chapters came about as a consequence of collaborative work that took place during this PhD. Finally, Chapter seven, the general discussion, presents a synthesis of all discoveries made during this project and discusses them in context. All data chapters included in this thesis have been written in the format of the journal that they were submitted to prior to thesis composition, although numbering has been amended where possible so that it is consistent throughout the thesis. Additionally, a reference list is included at the completion of each chapter for convenience.

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Chapter Two

Arrested embryonic development: A review of strategies to delay hatching in egg-laying reptiles

Anthony R. Rafferty[1] & Richard D. Reina[1]

[1]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

Published under the same name in Proceedings of the Royal Society B

Published 21st March 2012

Declaration for Thesis Chapter Two

Monash University

Declaration by candidate

In the case of Chapter two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, literature review and manuscript preparation	90%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Richard Reina	Supervision, manuscript editing and direction	10%

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Monash University, School of Biological Science, Clayton campus

	Signature	**Date**
Richard Reina	[redacted]	

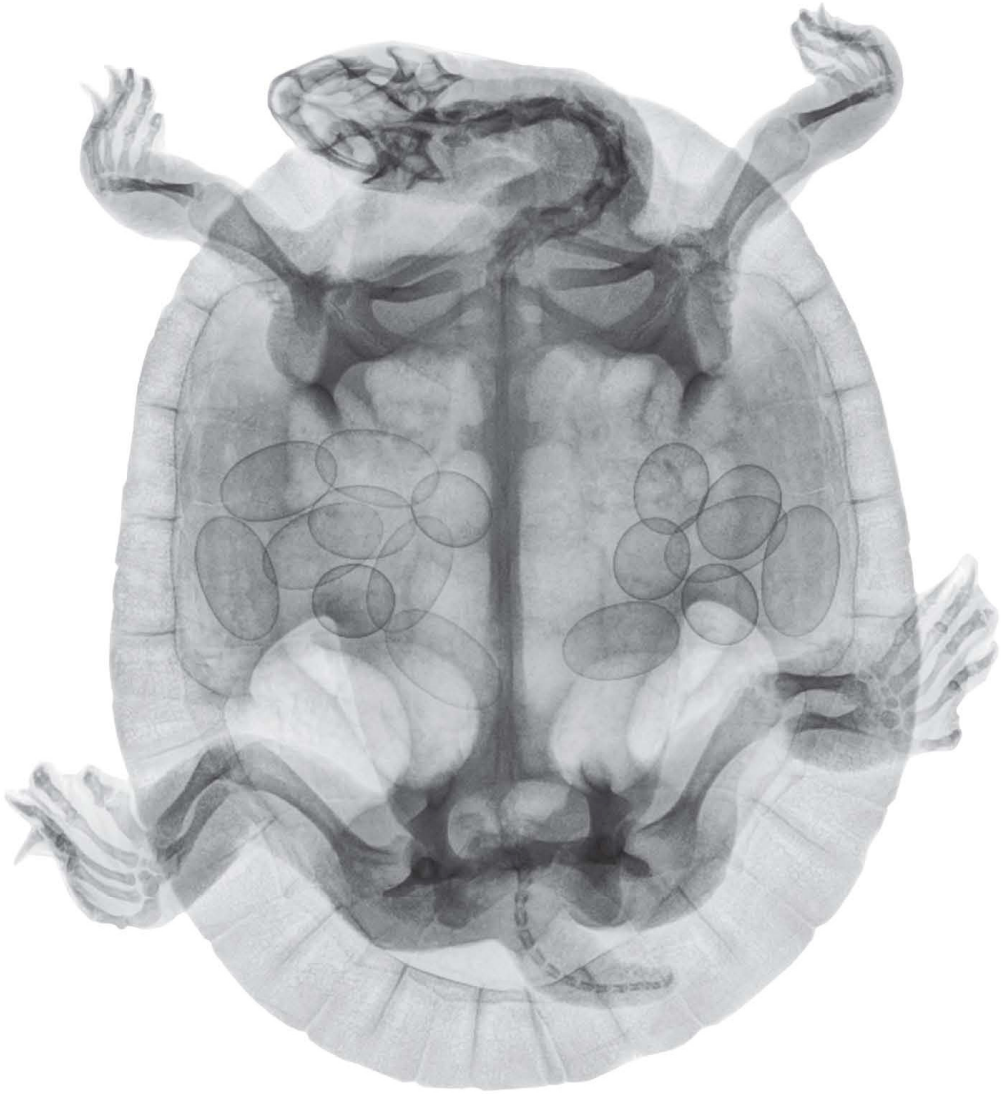
PROCEEDINGS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

ISSN 0962-8452

volume 279

number 1737

pages 2289–2496



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Proc Roy Soc journal cover; radiograph of a gravid Murray River turtle (*Emydura macquarii*)
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PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2012) **279**, 2299–2308
doi:10.1098/rspb.2012.0100
Published online 21 March 2012

Review

Arrested embryonic development: a review of strategies to delay hatching in egg-laying reptiles

Anthony R. Rafferty* and Richard D. Reina

Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

Arrested embryonic development involves the downregulation or cessation of active cell division and metabolic activity, and the capability of an animal to arrest embryonic development results in temporal plasticity of the duration of embryonic period. Arrested embryonic development is an important reproductive strategy for egg-laying animals that provide no parental care after oviposition. In this review, we discuss each type of embryonic developmental arrest used by oviparous reptiles. Environmental pressures that might have directed the evolution of arrest are addressed and we present previously undiscussed environmentally dependent physiological processes that may occur in the egg to bring about arrest. Areas for future research are proposed to clarify how ecology affects the phenotype of developing embryos. We hypothesize that oviparous reptilian mothers are capable of providing their embryos with a level of phenotypic adaptation to local environmental conditions by incorporating maternal factors into the internal environment of the egg that result in different levels of developmental sensitivity to environmental conditions after they are laid.

Keywords: amniotic; arrested development; ecology; evolution; reproduction; reptile

1. INTRODUCTION

Evolution of the amniotic egg was paramount in eliminating the need for vertebrate animals to reproduce in water, and allowed independent embryonic existence in the absence of parental care [1]. The amniotic egg was a major evolutionary adaptation that facilitated terrestrial exploitation, because on land, eggs are exposed to a highly variable environment in comparison with the relatively stable aquatic system [2]. In order to deal with potentially adverse environmental conditions, several lineages evolved ovoviviparity—the production of eggs that develop and hatch within the female's body, resulting in the birth of a relatively well-developed neonate [3], whereas those that retained oviparity (egg-laying) developed the ability to arrest embryonic development inside the egg until favourable conditions arise or return. Arrested embryonic development is an adaptation characterized by the downregulation or cessation of active cell division and metabolic activity of the embryo [4]. It is a reproductive strategy that is employed by many taxa including plants, insects and amniotic vertebrates, suggesting that it has evolved independently on numerous occasions [5]. This life-history trait confers a significant selective advantage because it allows embryos to respond to varying environmental conditions by altering their period of development [6].

Extant reptiles (squamates, crocodiles, turtles and the tuatara) possess the most diverse range of strategies to prolong the egg state, while in mammals and birds these mechanisms are less varied because of their higher level of parental care [4]. The incidence of developmental arrest decreases in mammalian species that provide more parental care, and bird embryos typically only retard development when parents are not tending to them [7–9]. The rich abundance of strategies to arrest development in reptiles enables embryos to withstand a changing incubation environment in a variety of ecological settings [10]. Most reptilian species undergo at least one period of developmental arrest while inside the amniotic egg, and in some cases both before and after oviposition [4,11], as summarized in figure 1.

Arrested embryonic development in mammalian species that provide parental input and care during embryonic development and after parturition has been reviewed extensively [5,6,12], but understanding of developmental arrest in oviparous reptiles that do not provide parental care after oviposition is limited. We present this information here and encourage future research to investigate the varying embryonic and early ontogenic strategies that are employed by different reptiles in response to their ecological demands.

(a) *The environmental influence on embryonic development*

Embryonic development occurs inside the amniotic egg (figure 2), which is composed of yolk and albumin encased in a porous, proteinaceous eggshell. The proteins produced in the mother's oviduct largely determine the egg environment, with yolk acting as the primary source of energy and albumin providing water and protein necessary for development [13,14]. The composition

* Author for correspondence (tony.rafferty@monash.edu).

Received 13 January 2012
Accepted 29 February 2012

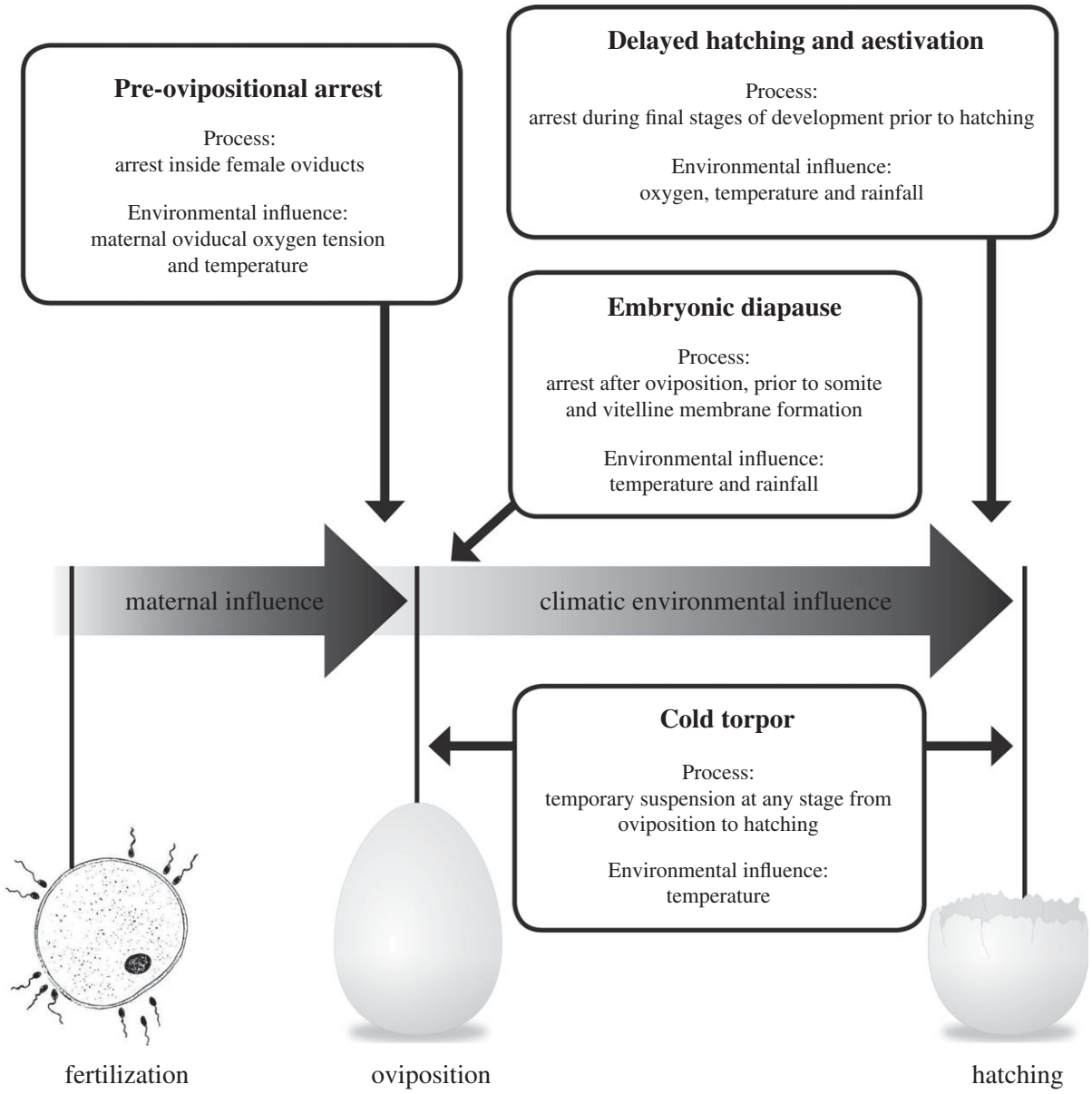

Figure 1. Summary of maternal and climatic environmental influences on the onset of arrested embryonic development between fertilization and hatching in oviparous animals that do not provide parental care after oviposition.

and function of the egg components change throughout the developmental period, and as a whole, the developing egg (embryo and its extra-embryonic membranes) interacts with the external environment [13,15].

In the oviduct, the external environment consists of an oviducal material that bathes the egg and fills the shell pores. Proteins have been identified in the oviducal fluid of some reptilian species including the estuarine crocodile, *Crocodylus porosus* [16]; the American alligator, *Alligator mississippiensis* [17]; and the gopher tortoise, *Gopherus polyphemus* [18], although there is still little known of the composition and function of the majority of oviducal products. The transfer of growth factors secreted by the oviduct of gravid *A. mississippiensis* has been shown to affect embryonic development after being taken up by the egg [13,19]. However, the significance of the transfer of such materials is not fully understood.

After oviposition, development is affected by the surrounding nest environment and many studies have documented an environmentally induced variation in development caused by factors such as temperature [20,21]. Reptilian embryonic development and hatchling phenotype are largely influenced by temperature and is known to determine sex in many species [22,23]. Temperature changes can affect the water potential of the egg and alter the metabolic rate and developmental period of a reptilian embryo [24]. This is evident through changes in embryonic oxygen consumption rates and the patterns of embryonic development [25,26].

2. SIGNIFICANCE OF ARRESTED DEVELOPMENT FOR OVIPAROUS REPTILES

Developmental arrest is a critically important reproductive strategy in the large range of egg-laying animals that provide no parental care after oviposition, because it provides their eggs a mechanism to respond to changing environmental conditions during embryonic development. From an ecological perspective, developmental arrest allows some embryonic resilience to adverse environmental conditions

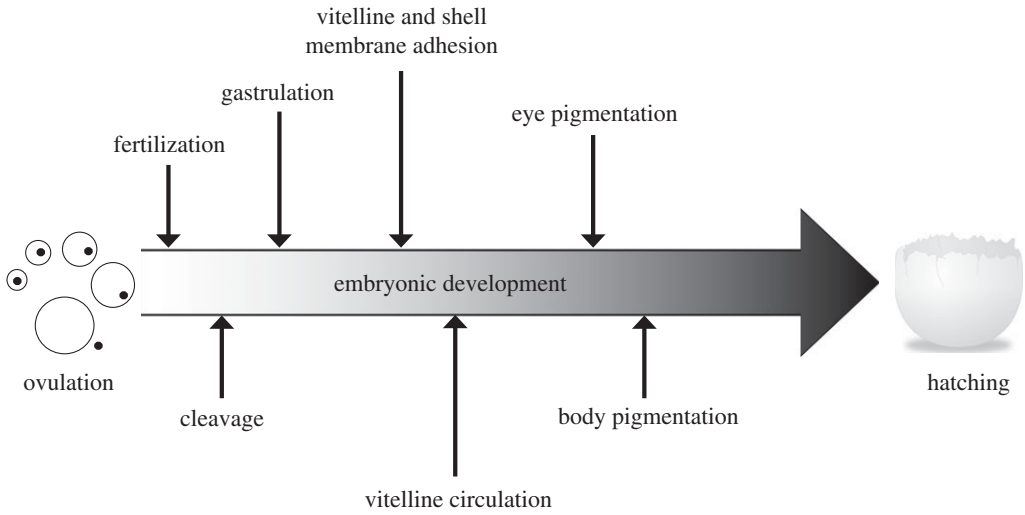

Figure 2. Chronology of the primary developmental events between ovulation and hatching inside the amniotic egg of a reptile.

that do not favour successful development, with the capacity to synchronize hatching with seasonal periods that benefit the resulting offspring. Although developmental arrest has been documented in various different species of reptiles, it is most widespread and better understood in chelonians [4,11,27–29]. Nevertheless, it is still generally poorly represented in the literature and requires considerably more investigation.

3. STRATEGIES TO ARREST DEVELOPMENT IN OVIPAROUS REPTILES

Many different types of arrest exist in animals, particularly reptiles, but they can all generally be classed as either endogenous or facultative [30]. Endogenous control of arrested development ensures that arrest occurs at the same developmental stage regardless of the surrounding environmental conditions. Facultative arrest on the other hand, comes about in direct response to environmental variables that do not favour successful development [30]. The following sections will outline each type of arrest in sequence beginning after fertilization of the egg and continuing through until hatchling of the egg in the nest (figure 1 and table 1).

(a) *Pre-ovipositional development and arrest*

Although the degree of embryonic development that has taken place prior to oviposition varies largely between and even within different reptilian species, all reptilian embryos will have advanced to, or beyond, the blastula stage in the oviduct before they are laid [50,59,60]. However, the embryos of some species enter an obligate developmental arrest, thus preventing development beyond a specific stage in the oviduct and ensuring that all eggs are laid at the same developmental stage. This is called pre-ovipositional arrest and persists only while the eggs are inside the female. This is not a developmental trait expressed by birds or crocodilians and if embryonic development in the oviduct extends beyond what is normally encountered for a species, it usually results in developmental deformities [4,61]. Pre-ovipositional arrest is therefore a way of protecting the embryo in an environment that would compromise survival if development were to continue.

Pre-ovipositional arrest is generally observed during early embryogenesis but there are situations where it arises late in development. The tuatara, *Sphenodon punctatus*, and the common chameleon, *Chamaeleo chamaeleon*, are capable of arresting embryonic development during gastrulation, in addition to all marine and freshwater turtles that enter pre-ovipositional arrest as mid and late gastrulae, respectively, [11,28,40,62,63]. In contrast, squamate species including the North American iguanids, *Urosaurus ornatus* and *Sceloporus undulatus*, and the Indian agamid, *Calotes versicolor*, are capable of extended periods of embryonic development in the oviducts and embryos will arrest development if hatching is imminent and the eggs have not been laid [21,64].

(i) *Causes of pre-ovipositional arrest*

There are several morphological and physiological constraints likely to have given rise to pre-ovipositional arrest and the need to lay eggs at specific, mostly early, stages of development. In turtles, crocodilians and the tuatara, limited oxygen exchange might occur in the oviduct as a result of shell calcification and the shell pores filling with oviducal fluid [21]. Pre-ovipositional calcification of turtle eggs becomes complete when the embryos reach gastrulae and enter pre-ovipositional arrest approximately 7 days after ovulation [31]. Similarly, calcification of the eggshell is extended in squamate species that arrest during late developmental stages and completes just prior to arrest [65]. Eggshell calcification may infer a restriction on respiratory gas exchange needed for further growth of the embryo [32,33]. A reduction in eggshell thickness has been associated with extended egg retention and advanced embryonic development in reptiles, presumably because the availability of O_2 to the embryo is enhanced [31,66]. However, instances of eggshell thickening associated with extended egg retention in turtles have also been reported [67]. Additionally, the eggshell provides a source of calcium for developing turtle embryos and reducing the degree of eggshell calcification in order to achieve greater O_2 exchange may decrease embryo fitness [21,68].

The embryonic vitelline membrane and inner shell membranes of the egg must adhere to one another after calcification of turtle and crocodilian eggs to allow gas

Table 1. Literature summary of the strategies to arrest embryonic development in oviparous animals in response to different environmental variables.

type of embryonic developmental arrest	environmental influence	literature reference
pre-ovipositional arrest	oxygen	[21,31–39]
	temperature	[29]
embryonic diapause	temperature	[4,11,25,29,40–46]
	rainfall	[4,11,39,41–43,45–49]
cold torpor	temperature	[4,7,8,11,50–52]
delayed hatching and aestivation	oxygen	[53,54]
	temperature	[4,11,41,54,55]
	rainfall	[41,53,54,56–58]

diffusion and embryonic growth. Membrane fusion can only occur after oviposition because it is likely that the connection would rupture when eggs are jostled during oviposition, leading to embryonic mortality [21,69]. Vertical or horizontal movement of turtle eggs after membrane adhesion can cause movement-induced mortality [69–71], which has also been demonstrated in crocodilian embryos between 2 and 9 days after oviposition [22,61]. The presence of viscous oviducal secretions surrounding the egg and filling its pores inhibits formation of a respiratory surface that allows efficient gas diffusion [34,72].

Currently, there are no published data supporting the suggestion that hypoxia within the reptilian oviduct induces pre-ovipositional arrest [21,35,36]. Intra-oviducal oxygen tensions also remain unknown in reptiles, although there have been studies that estimated the oxygen tension *in utero* of the eastern fence lizard, *Sceloporus undulatus*, to be hypoxic [37,38]. Hypoxia is known to induce developmental arrest in budding yeast, *Saccharomyces cerevisiae*, and embryos of the nematode *Caenorhabditis elegans*, zebrafish, *Danio rerio*, and mouse, *Mus musculus* [73–76]. Hypoxia also maintains arrest in several species of short-lived fishes [77]. Oviducal oxygen tension has been measured at 37 mm Hg (5.3% O_2) during the formation of pre-implantation blastocysts in pregnant hamsters [78]. These comparative findings suggest that a hypoxic environment within the oviduct of oviparous reptiles is likely and that a lack of oxygen may induce and/or maintain pre-ovipositional arrest.

Hypoxia causes the upregulation of the insulin-like growth factor binding protein (IGFBP-1) [79–81] that binds to insulin-like growth factors (IGFs) and inhibit their activity. IGFs are greatly involved in embryonic metabolic activities and regulate all aspects of development and growth [82]. Altered expression of IGF proteins affects embryonic development in guinea pigs [83] and binding of IGFBP-1 to IGFs during hypoxia inhibits growth and causes developmental arrest in zebrafish embryos [84]. It is unknown whether this occurs in reptilian embryos that experience pre-ovipositional arrest, but it is plausible that oviducal hypoxia may cause expression of IGFBP-1 during certain developmental stages, leading embryos to arrest in the oviducts. After oviposition, increased oxygen levels or natural degradation of IGFBP-1 may then allow IGF activity and resumption of development.

IGF-I and IGF-II have been discovered in the albumin of shelled eggs present in the oviducts of the American alligator, *A. mississippiensis* [85], and peak IGF levels have been recorded in gravid females of this species [19,86]. The maternal influence over developmental arrest is widely reported in mammals [6], but is a topic that is relatively unexplored in reptiles. Maternal effects, defined as the underlying pressure exerted by the maternal genotype or phenotype that influences the offspring phenotype [87], have been linked to hatching success, hatchling growth rate and righting response in the snapping turtle, *Chelydra serpentina*, despite the absence of the mother during incubation [88,89]. In addition, some female leatherback turtles, *Dermochelys coriacea*, are more successful mothers than others [90,91] and it is therefore plausible that female reptiles directly influence the development of their embryos inside the oviduct with such influence possibly persisting after oviposition.

(b) *Post-ovipositional development*

Eggs that are laid in a state of pre-ovipositional arrest generally recommence active development shortly after oviposition in the absence of subsequent types of post-ovipositional arrest [11,28]. Resumption of normal development after oviposition usually occurs within a matter of hours if environmental factors are satisfactory [62,92]. Early stage embryonic development in turtle and crocodilian eggs involves liquefaction of the egg albumin and the transfer of water to the vitelline sac in order to cause the formation of the subgerminal space [93–97]. The vitelline sac also begins to expand inside the egg, causing the vitelline membrane to come in contact with the inner shell membrane. At the point of adhesion of these two membranes, a white spot forms on the surface of the eggshell which closely precedes chalking (whitening) of the eggshell. The white spot generally develops between 1 and 4 days after oviposition and is the first external visual sign of the active development that is taking place inside the egg [4,72].

(i) *Post-ovipositional arrest*

Both eggshell type and geographic location have been used to predict the likelihood of a species possessing developmental arrest after oviposition [4,11,41]. Eggshell thickness is a primary focus in the debate surrounding the evolution of developmental arrest and viviparity in reptiles, and it is known to affect aspects of embryonic physiology [98]. Species that lay thinner-shelled pliable eggs typically do not use developmental arrest after oviposition and as a result they have shorter incubation periods than those laying thicker, more rigid, brittle-shelled eggs that do use it [11]. In addition, seasonal climatic effects influence the occurrence of developmental arrest in different geographic locations and it is likely to occur in reptilian species nesting in regions with distinct seasons and locations that are characterized by a warm temperate or tropical climate [30].

In sub-tropical and tropical regions, arrested development in chameleons is closely related to seasonal temperature fluctuations [4]. This is also the case for turtle species that nest during temperate autumn, although embryonic development does not appear to arrest during

egg incubation when nesting occurs during temperate spring and summer in ephemerally suitable habitats [42]. On the other hand, developmental arrest in turtle species that nest in tropical and sub-tropical regions is most likely dictated by the seasonality of rainfall rather than temperature [4,11,42]. For example, pre-ovipositional arrest persists in embryos of the northern long-necked turtle, *Chelodina rugosa*, when eggs are laid underwater during the wet season. Embryos remain as gastrulae in response to underwater hypoxia until they are exposed to atmospheric oxygen when the nests dry out during the dry season [43]. A modified vitelline membrane in the eggs of this species prevents excessive water uptake during submersion [39]. The entire ontogeny of *C. rugosa* can be linked to environmental parameters with rainfall and hypoxia being involved in not just pre-ovipositional arrest in early embryos [99].

(ii) *Post-ovipositional embryonic diapause*

Embryos from several chelonian and chamaeleonid lizard lineages undergo a period of post-ovipositional embryonic diapause while they are still gastrulae [42,100]. Embryonic diapause is observed in the common chameleon, *C. chamaeleon*, the veiled chameleon, *C. calyptratus* and the Indian chameleon, *C. zeylanicus*, which lay eggs during temperate and subtropical autumn that take up to a year after oviposition to hatch because they arrest during cold winter months [40,44,101]. Embryos of the jewelled chameleon, *Furcifer lateralis* and the panther chameleon, *F. pardalis*, may also experience periods of embryonic diapause during the cooler dry season because they are laid during the warm wet season and do not hatch until the following wet season [45,46,47,102]. Embryonic diapause is an obligate part of the life cycle for each of these species, although resumption of development is facultative and does not appear to be linked to a mandatory environmental cue like those needed during embryonic diapause in some turtle species [29]. However, the length of time that embryonic diapause persists after oviposition has been linked to temperature in some chameleon species [29,40,101].

Australian broad-shelled turtle, *Chelodina expansa*, embryos also typically remain arrested after oviposition for several weeks before recommencing development briefly and entering a secondary state of embryonic diapause prior to somite and vitelline development, which persists throughout the winter period [29,42]. This type of embryonic diapause occurs after white spot development and is seen in healthy embryos that are in an environment which would normally promote active development [4]. Embryos in this state need to be exposed to an obligatory trigger to recommence development and in the absence of this cue, embryos remain arrested and eventually die [4].

Freshwater turtle embryos that show embryonic diapause take longer to reach specific development stages than those that do not when incubated at the same temperature [4]. The occurrence of embryonic diapause can be identified by comparing the developmental schedules of siblings or embryos of closely related species and recognizing whether pre-somite development is extended in relation to the remainder of development (i.e. whether development occurs in the expected timeframe) [4]. Five developmental stages are used to draw comparisons based on a determined developmental series [103]. These include the appearance of blood islands (stage 5), development of the haemodisc (vitelline circulation at stage 8), pigmentation of the eye (stage 12), pigmentation of the body (stage 20) and hatching (stage 26). The appearance of the white spot has also been used as a defining developmental stage [72,100]. These aforementioned stages can be observed in many species through non-destructive candling techniques and so make development in viable eggs relatively simple to assess [104].

In warm temperate regions, where oviposition generally occurs in autumn, embryos enter a state of embryonic diapause during the winter months and for some species such as the striped mud turtle, *Kinosternon baurii*, it has been described as seasonally dependent [4,42]. *Kinosternon baurii* females nest twice yearly during both spring and autumn, but embryos from the same females that are laid during both seasons only express embryonic diapause during autumn, suggesting that the occurrence of this reproductive characteristic is dependent on some external cue [42]. These embryos require a period of chilling to terminate embryonic diapause and resume development to hatching. Unchilled eggs of *D. reticularia*, the ornate wood turtle, *Rhinoclemmys pulcherrim*, and the Indian black turtle, *Melanochelys trijuga*, also require this stimulus, and they will remain arrested and subsequently die in its absence [11,25]. The same is true for *C. expansa* embryos, which require a chilling period during their developmental cycle in order for eggs to recommence development after embryonic diapause [29].

The 'decision' of whether or not an embryo expresses embryonic diapause is thought to occur during late oogenesis (between ovulation and fertilization) in *K. baurii* [42]. If females underwent chilling during this period, their embryos did not express embryonic diapause post-oviposition, but the embryos of unchilled females did [42]. This suggests that expression of embryonic diapause in embryos is determined by the temperature that mothers experience during late oogenesis, supporting the theory that maternal experience may influence the phenotype of their young [88,89].

The presence of embryonic diapause is characterized by annual wet–dry seasonality rather than by the temperature in sub-tropical and tropical locations [41]. These regions have seasonal rainfall patterns with minimal annual variation in temperature. In these areas, early incubation is spent in embryonic diapause during the wet season and the beginning of the dry season with hatching occurring the following wet season. In Pacific coastal regions of Mexico and Central America, turtle species including *Kinosternon*, *Staurotypus* and *Rhinoclemmys* express embryonic diapause in relation to seasonal rainfall, the same is also true for the north Australian snapping turtle, *Elseya dentata*, that lays its eggs during the austral wet season [42,105]. By laying eggs during the wet season, reptilian females may free themselves from the burden of carrying eggs for extended periods until suitable nesting environments are available. Additionally, it may also allow them to exploit the abundant food resources existing during these periods and thereby to produce subsequent clutches, assuming that successive clutches are not ovulated immediately after oviposition [43]. Likewise, hatching emergence during wet seasons may infer an advantage for the hatchlings because prey abundance is greater [48].

(iii) *Cold torpor*

Cold torpor is the facultative suspension of development that embryos use to survive brief periods during temperatures that are too low to support developmental requirements [4,11,50]. The embryos of most species of bird and reptile are capable of using this type of arrest at any stage during incubation, although others such as the montane lizard, *Acritoscincus duperreyi*, can only enter cold torpor when they are full-term embryos and not during earlier stages of development [49]. It is a direct response to unfavourable weather and in many cases, it protects embryos for varying durations at below-critical temperatures, although the chances of mortality increase with increased duration spent in torpor [4,51]. Avian species can typically only endure several days in torpor, whereas reptile embryos can withstand weeks [4]. Cold torpor can maintain pre-ovipositional arrest in turtle embryos [52] and in avian species that do not use pre-ovipositional arrest, torpor can prevent development of embryos after oviposition until all eggs in the clutch have been laid [8]. This allows the synchronization of development and hatching of the clutch [7].

(iv) *Delayed hatching and embryonic aestivation*

Delayed hatching and embryonic aestivation are strategies employed to prolong the residence of an embryo within the egg in response to adverse environmental conditions [4,42]. During both forms of arrest, the embryo is in the final stages of development and typically remains within an unpipped egg, although some species of bird may pip the egg before delaying hatching for up to a week [4,106]. Delayed hatching and aestivation differ only in the duration and degree of metabolic downregulation, so they are generally not distinguished in the literature. Delayed hatching occurs in avian, crocodilian, squamate and chelonian groups and although it may persist for several weeks in reptile eggs, it is usually brief in birds [4,55]. In contrast, aestivation is a late-embryonic dormancy that may last for weeks or months and empirical evidence of its occurrence has only been documented in turtles, although it may also occur in crocodiles and lizards [55,56,57,107]. Both delayed hatching and aestivation allow protection of young during unfavourable conditions and also permit synchronization of hatching when environmental surroundings promote optimum survival [4,53,57].

Generally, approximately 2 days before a turtle embryo pips the shell, the amnion ruptures inside the egg and the chorio-allantois moves posterior to the embryo, revealing the head and forelimbs and so freeing the embryo to pip the shell [11]. At the time of hatching, a yolk sac will also be external to the plastron [4,108]. However, when an embryo delays hatching, there is usually a greater delay in pipping after rupture of the amnion and movement of the chorio-allantois, in addition to internalization of the yolk reserve. Yolk absorption and final assimilation of any blood from the chorio-allantois signal the completion of embryonic development in turtles and crocodilians.

Turtle species including the pig-nosed turtle, *Carettochelys insculpta*, *K. scorpioides* and *K. flavescens* gradually enter aestivation after the chorio-allantois parts and migrates, such that it no longer covers the body of the turtle inside the eggshell [4]. Aestivation is sometimes considered an extension of delayed hatching if optimal conditions for hatching do not arise. The prolonged incubation period associated with aestivation generally involves the downregulation of metabolic processes and the subsequent decline in oxygen consumption rates to extremely low but steady levels [4]. Aestivation can extend for periods up to 116 days in *K. scorpioides* and 232 days in *K. flavescens* embryos if a stimulus to pip remains absent. In the former species, oxygen consumption can decrease to approximately 22 per cent of the peak rate [4]. Oxygen consumption rates of *C. insculpta* can decline to one-third of the peak rate when experiencing delayed hatching and aestivation at 30°C [109]. During these periods, the fully developed mature hatchlings of *C. insculpta* can remain within the egg for up to 59 days, after which the yolk reserve becomes depleted [57,109]. Extended aestivation periods potentially result in the weakening of the embryo and increase the likelihood of mortality if all energetic reserves have been exhausted and a stimulus to pip is still absent [4,41]. Not all species of turtle are capable of aestivation and when late-term embryos of the painted turtle, *Chrysemys picta* and *C. serpentina* are exposed to conditions that induce aestivation in other aestivating species, development and hatching are hastened because of the unfavourable conditions [4].

The proximate mechanisms associated with the metabolic changes observed during delayed hatching and aestivation, which appear to be actively regulated independent of temperature, are poorly understood. However, a possible factor involved in the process may be thyroid hormone [54,110]. Thyroid hormone is vital for the growth and function of most vertebrate tissues and is capable of acting on both metabolic and non-metabolic pathways to influence embryonic tissue accretion and differentiation [111]. Thyroid hormone is also involved in energy metabolism and has been linked to the hatching process in chickens [112,113]. Increased thyroid hormone levels are known to stimulate hatching, and production of this hormone is induced in hypoxic conditions [112]. Hypoxia is the proximal cue known to trigger aestivating *C. insculpta* eggs to rapidly hatch, although whether this process involves thyroid hormone remains to be tested [57].

Environmentally induced cues have been explained to terminate delayed hatching and aestivation. Both processes were originally thought to occur in response to hot environments, although recent research suggests that delayed hatching and aestivation are more likely strategies to coincide hatching with wet season productivity [41,57]. In doing so, delayed hatching and aestivation allow hatchlings to exploit early wet-season productivity to promote growth and survival rather than to avoid any embryonic stress that might be caused by late dry-season conditions [4,106,109]. As mentioned previously, hypoxia induced by complete water inundation of *C. insculpta* eggs is the trigger that induces explosive hatching during periods of heavy rainfall [57,109]. Eggs of hole-nesting crocodilians may also undergo this process based on similar nesting and hatching patterns, but more research is needed to confirm this [107]. Additionally, heavy rainfall at the onset of the wet season may trigger hatching in the Fiji-crested iguana, *Brachylophus vitiensis*, but it has not yet been determined whether extended incubation periods are associated with delayed

hatching and aestivation, or embryonic diapause in this species [55,114].

Evidence is also emerging which suggests that there are additional mechanisms, other than fluctuations in nest gas concentration, that trigger eggs to hatch synchronously after undergoing a period of quiescence. Vibration-induced hatching, triggered by the vibrations of some eggs hatching earlier than others, has been proposed [55]. In addition, audible triggers such as the pipping of eggs or vocalizations by embryos is plausible considering that sibling communication has been identified in eggs of both crocodilian and some avian species [54,58]. However, all of these hypotheses remain to be fully tested.

4. CONCLUSIONS AND DIRECTIONS FOR FUTURE ENQUIRY

All forms of arrested development synchronize the developmental timing of turtles in accordance with conditions that increase the chances of embryonic survival in the absence of parental care [115]. Developmental arrest allows turtles to nest for longer periods throughout the year and also permits hatchling turtles to emerge when food may be more abundant. Early stage pre-ovipositional arrest guarantees that embryos avoid movement-induced damage during oviposition and ensures that eggs are laid prior to the resumption of development. Pre-ovipositional arrest is universal in turtles and occurs in many other species of reptile, but it is the least studied form of arrest. The majority of studies investigating strategies to arrest embryonic development have focused on the time between oviposition and hatching, thus overlooking the period of development inside the mother. The evidence presented in this review suggests that the conditions experienced by the embryo while inside the oviduct influences the ability of the embryo to respond to environmental factors including oxygen tension, temperature and moisture. The key to fully understanding developmental processes includes addressing maternal influences and priority should be placed on understanding how the relationship between maternal factors and environmental factors affect embryonic development.

Embryonic diapause, cold torpor, and delayed hatching and aestivation allow avoidance of unfavourable environmental conditions that would otherwise have a negative effect on successful growth and development of the embryo. The majority of work on these topics has been undertaken in an artificial environment in the laboratory and more field-based research is needed to fully understand how processes such as developmental arrest are influenced by natural environments. In addition, the developmental chronology of many oviparous animals remains unknown and by identifying how embryos develop, comparisons and conclusions can accurately be drawn between groups that employ different developmental strategies to deal with a changing environment. Furthermore, future investigations should identify whether embryos will preferentially arrest at certain developmental stages more than others.

Although the assumption that developmental arrest occurs in response to environmental factors associated with an embryo's external environment is widely held, the cues that cause the onset or cessation of arrest still remain unclear in most cases. Future studies in the field of reptilian embryology need to identify how environmental ecology can dictate the evolution of developmental processes associated with embryonic development and ontogeny [116,117]. In doing so, we will better understand how the natural environment has shaped the evolutionary history of a wide range of species and help uncover some of the secrets associated with terrestrial invasion and evolution of the incredible ecological adaptations that have come about in response to challenges associated with laying shelled eggs on land [118].

Overall, the topic of embryonic developmental arrest warrants much more attention than it has previously received. Understanding the processes involved in arrest has implications for conservation biology and population recovery of critically endangered egg-laying animals because it is essential to understand the natural history of a species before attempts can be made to artificially incubate eggs successfully. In many captive situations, it is unlikely that eggs will survive to hatching if the triggers for breaking periods of arrest are unknown. In addition, understanding what induces a state of early stage embryonic arrest may allow the successful transport of eggs without risk of movement-induced mortality, thus allowing for *ex situ* research on a multitude of different species. Finally, it also offers a fundamental understanding of the diversity of reptilian developmental processes and their consequences.

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Chapter Three

Embryonic death is linked to maternal identity in the leatherback turtle (*Dermochelys coriacea*)

Anthony R. Rafferty[1, 2], Pilar Santidrián Tomillo[3], James R. Spotila[3], Frank V. Paladino[4] & Richard D. Reina[2]

[1]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia
[2]School of Biological Sciences, Monash University, Melbourne, Victoria, Australia
[3]Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania, United States of America
[4]Department of Biology, Indiana - Purdue University, Fort Wayne, Indiana, United States of America

Published under the same name in PLoS ONE
Published 14th June 2011

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In the case of Chapter three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, initiation, design, data analysis and manuscript preparation	70%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Pilar Santidrian Tomillo	Data collection and manuscript editing	10%
James Spotila	Manuscript editing	5%
Frank Paladino	Manuscript editing	5%
Richard Reina	Supervision, manuscript editing and direction	10%

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

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Pilar Santidrian Tomillo	[redacted]	
James Spotila		May 10, 2012
Frank Paladino		May 12/2012
Richard Reina		

Measuring the carapace length of a leatherback turtle (*Dermochelys coriacea*)
Photo taken by Lisa C. White

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Embryonic Death Is Linked to Maternal Identity in the Leatherback Turtle (*Dermochelys coriacea*)

Anthony R. Rafferty[1]*, Pilar Santidrián Tomillo[2], James R. Spotila[2], Frank V. Paladino[3], Richard D. Reina[1]

1 School of Biological Sciences, Monash University, Melbourne, Victoria, Australia, **2** Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania, United States of America, **3** Department of Biology, Purdue University, Fort Wayne, Indiana, United States of America

Abstract

Leatherback turtles have an average global hatching success rate of ~50%, lower than other marine turtle species. Embryonic death has been linked to environmental factors such as precipitation and temperature, although, there is still a lot of variability that remains to be explained. We examined how nesting season, the time of nesting each season, the relative position of each clutch laid by each female each season, maternal identity and associated factors such as reproductive experience of the female (new nester versus remigrant) and period of egg retention between clutches (interclutch interval) affected hatching success and stage of embryonic death in failed eggs of leatherback turtles nesting at Playa Grande, Costa Rica. Data were collected during five nesting seasons from 2004/05 to 2008/09. Mean hatching success was 50.4%. Nesting season significantly influenced hatching success in addition to early and late stage embryonic death. Neither clutch position nor nesting time during the season had a significant affect on hatching success or the stage of embryonic death. Some leatherback females consistently produced nests with higher hatching success rates than others. Remigrant females arrived earlier to nest, produced more clutches and had higher rates of hatching success than new nesters. Reproductive experience did not affect stage of death or the duration of the interclutch interval. The length of interclutch interval had a significant affect on the proportion of eggs that failed in each clutch and the developmental stage they died at. Intrinsic factors such as maternal identity are playing a role in affecting embryonic death in the leatherback turtle.

Citation: 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(6): e21038. doi:10.1371/journal.pone.0021038

Editor: Howard Browman, Institute of Marine Research, Norway

Received February 20, 2011; **Accepted** May 17, 2011; **Published** June 14, 2011

Funding: Financial support was provided by the Betz Endowment of Drexel University, the Earthwatch Institute (www.earthwatch.org), and The Leatherback Trust (www.leatherback.org). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Anthony.Rafferty@sci.monash.edu.au

Introduction

Leatherback sea turtles (*Dermochelys coriacea*) are large, pelagic reptiles that undertake long oceanic migrations [1,2,3]. They were once thought to be the most abundant of the 7 living sea turtle species but have suffered substantial population declines in many parts of the world [4]. The reduction in the global leatherback population has been estimated at ~67%, with eastern Pacific colonies suffering a 90% decrease in the last two decades [5,6]. As a result, the population at Parque Nacional Marino Las Baulas (PNMB) in Costa Rica currently faces extinction [6,7].

Declining leatherback numbers have been attributed to anthropogenic factors such as fishing practices causing unsustainable adult mortality, egg poaching on nesting beaches, and habitat degradation [6,8,9]. Natural processes such as beach location, tidal inundation, bacterial and fungal attack and low hatching success are also thought to play a role in their demise [10,11,12,13,14]. Leatherbacks are now classified as critically endangered by the Species Survival Commission (IUCN)[15] and are the only extant species within the family Dermochelyidae.

Leatherback females return to their natal beaches to nest every 3–4 years once sexually mature [16,17] to lay an average 7 clutches of eggs during a nesting season. Nesting success and hatchling output varies within and between nesting seasons [18]. Generally during a nesting period, a gravid female will return every 9–10 days to lay an average of 65 eggs per clutch. [17,19]. They are unusual among oviparous amniotes because they have the highest clutch frequency and produce the biggest mass of eggs during each reproductive cycle using an evolutionary strategy thought to overcome low hatching success [20]. Leatherbacks have an average global hatching success rate of ~50%, lower than all other marine turtle species, which have hatching success rates in excess of 80% [10,16]. Low hatching success is an artefact of reduced genetic diversity in declining avian populations [21]. However, this is unlikely the case for leatherback turtles because the worlds largest stable and increasing leatherback rookeries in Western Africa [22,23] still have relatively low hatching success rates (33–39% at Awala–Yalimapo beach in French Guiana [24]; 10.6–56.0% in Suriname [25] and 67–69% in Gabon [26,27]).

Infertility causes low hatching success in some avian species [28,29]. However, this is not the case for leatherback turtles, with low hatching success attributed to high levels of early stage embryonic death [10]. At PNMB embryonic death has not been linked to extrinsic environmental factors that include temperature and oxygen levels within relocated nests [30,31]. However, in natural nests environmental variables such as precipitation and temperature are believed to play a role (Santidrián Tomillo et al. unpublished data), [18]. The high levels of variation and low coefficients of the relationships between embryonic death and temperature in previous studies suggest that factors other than

temperature are involved [18]. Investigations including intrinsic factors such as the effects of maternal identity are only cursory and need further investigation [18,20,31]. However, it is known that some leatherback females are better mothers than others [10].

Maternal effects are defined as the underlying pressure exerted by the maternal genotype or phenotype that influences the offspring phenotype [32]. Maternal reproductive investment of turtles is directly represented by the allocation of energy and nutrients because there is no post-hatching parental care [33]. During reproduction, females produce amniotic eggs that consist of yolk and albumin encased in a proteinaceous shell. Approximately 7 days after ovulation, sea turtle embryos enter a state of pre-ovipositional developmental arrest when they become gastrulae inside the oviducts of the female [34,35]. The mechanisms that cause arrest are currently unknown. Arrest persists for the remaining period of egg retention and ensures that membrane adhesion does not occur inside the egg prior to oviposition, thus preventing movement-induced mortality during laying [36]. Bell et al. (2003) found that the majority of leatherback embryos dying were at Miller's (1985) stage 6 (oviposition) of development. This suggests that embryos died as arrested gastrulae before they were laid or because they failed to break arrest and continue developing afterwards.

Gastrulation is evidently a very important developmental checkpoint for many species laying cleidoic eggs. In some reptilian species, if embryos have to remain as arrested gastrulae during periods of extended egg retention it can result in developmental deformities and even death of the embryos [37]. In addition, pre-ovipositional death of avian embryos occurs if egg development exceeds, or has not reached gastrulation at the time of oviposition [28].

Extended egg retention and hence periods of arrest during interclutch intervals have been recorded at 63 days in the olive ridley turtle (*Lepidochelys olivacea*) [38]. Other species of marine turtle have shorter interclutch intervals and therefore embryos are in arrest for a shorter time. Leatherback interclutch intervals range from 7–14 days [17], with older females having shorter interclutch intervals than younger or first-time nesters (Reina, unpublished data). This raises the question whether the duration of retention and arrest has an affect on embryo survival and if the reproductive experience of the female may also play a role.

The objectives of this study were to investigate whether the maternal identity, the year that each female nests, the position of each clutch relative to other clutches laid that season and the time of nesting each season were affecting hatching success and stage of death of embryos. In addition we wanted to uncover whether there was a difference in hatching success levels and stage of death of embryos between remigrants and new nesters, and between females with different interclutch intervals. Identifying the main cause of early stage embryonic mortality in leatherback nests at PNMB will not just tell us more about the life history patterns of these animals, but also offer an insight into the developmental processes occurring inside the cleidoic egg. This will prove beneficial to those studying reduced hatching success in reptiles and birds. In addition, it may be possible to increase hatching success rates by improving early stage embryonic survival and therefore increase the number of hatchlings entering waning populations.

Methods

Ethics statement

This study was conducted under MINAET permits (ACT-OR-052; ACT-OR-051; ACT-PNMB-010-2008; ACT-PNMB-009-2008) and was approved by the Animal Care Committee of Drexel University (IACUC: 18467; 16532).

Study site and data collection

Data for this study was collected as part of a continuing beach monitoring project at Parque Nacional Marino Las Baulas in the Guanacaste province of Costa Rica (10°20′N, 85°51′W). We used the results of nightly beach surveys conducted during five nesting seasons between October and February from 2004/05 to 2008/09. Approximately 90% of all nesting activity was encountered during these patrols and nesting data was collected for each female using methods previously described [17,39]. Females were identified as new or remigrant by the absence or presence of PIT tags applied in a previous nesting season. Untagged turtles had a tag applied into the muscle of the shoulder using standard techniques [40]. Oviposition was observed during successful nesting events, eggs were counted and nests were marked with a thermocouple as eggs were laid. The nest location was triangulated to enable subsequent excavation for determination of developmental success.

Staging dead embryos

Each recorded nest was excavated two days after first hatchling emergence and the contents of all unhatched eggs were examined. The dead embryos were staged using the field-staging method developed by Leslie et al. (1996), which relates to specific developmental stages of Miller's (1985) 31-stage developmental chronology for marine turtles. This criterion corresponds to the presence or absence of an embryo and its associated morphology [10,30]. Using these guidelines, "field stage 0" was classified as Miller's (1985) stage 12 and below with no embryo or blood vessels visible, with a maximum period of growth of 4 days post oviposition. "Field stage 1" was between Miller's (1985) stages 13–16 and was identified by the presence of an unpigmented embryo <10 mm in length with blood vessels visible, with period of growth from 4–9 days. "Field stage 2" was between Miller's (1985) stages 17–23 and was determined from the presence of an embryo 10–20 mm in length with pigmented eyes, with period of growth from 9–24 days. "Field stage 3" was between Miller's stages 24 and 31 and included an embryo >20 mm long that was fully pigmented, with growth time from 24–60 days. Some previous studies also assigned eggs to an "unknown" category due to decomposition [10]. However for the purpose of this study, any decomposing eggs not exhibiting an embryo or evidence of blood vessel development within the egg was classified as field stage 0 based on the definitions outlined above. Therefore no eggs were assigned to an unknown category for this study.

Data analysis

Females nesting more than once each season were included in the analysis. Hatching success was calculated as a percentage of the total number of eggs laid in each clutch. Embryonic mortality at each field stage was calculated for each clutch as a percentage of the total amount of eggs that failed in the clutch. Clutch position was identified as its numerical arrangement within successive clutches laid by each specific female in a single season (i.e. clutch position of the first recorded clutch of the season was 1, the second clutch laid by that same female was 2, and so on). Females' reproductive experience was defined as either 'new' or 'remigrant' from the PIT tag records. The interclutch interval (number of days) between successive clutches for each female was determined using the methods outlined by Reina et al. (2002). Data was included in the analysis if the interclutch interval was between 7–13 days (inclusive). Successful ovulation and full calcification of the eggshell takes a minimum of 6 days [35], so it is not possible for females to lay successive clutches in less than 7 days. In addition, it is possible that females may nest twice within a 14-day period based on this assumption. Therefore, the longest interclutch

interval considered was 13 days, with interclutch intervals greater than this likely resulting from an intervening, unobserved nest. The time of nesting during each season was calculated using weekly 7-day intervals beginning when the first nest that season was laid. Female arrival time was defined as the nesting week in the season when each female laid her first nest.

In order to determine whether embryonic mortality occurred equally at all stages the mean log transformed percent mortality values were compared between each field stage using a one-way analysis of variance (ANOVA) and a Tukey's HSD test. Linear mixed effect models (using the 'LME' function in the 'nlme' package of R statistical package) were used to test the effects of female identity, season, clutch position and time of nesting on various different variables. They were most appropriate because they identify the variation that exists between and among variables such as female identity [20,41,42]. Female identity, clutch position and time of nesting were treated as random effects in each model. Hatching success, clutch number and clutch size were treated as response variables, while season, interclutch interval, reproductive status and stage of embryonic death were treated as fixed predictor variables. Models were compared using the Akaike Information Criterion (AIC). P-values and the log-likelihood test statistic (denoted as 'L' in text) are reported for these tests. Linear regression was also used in addition to the mixed model to show the relationship between interclutch interval and percent hatching success. The coefficient of determination (R^2 value) is reported for this test to allow interpretation of variation explained by the mixed model. Finally, ANOVA was used to identify whether female reproductive status affected initial arrival time of females each season and the number of clutches laid.

All data analysis was conducted in R, statistical package 2.11.0 (R Development Core Team 2010) using log transformed percent values. Statistical significance was accepted at the 0.05 level and all values are reported as the mean ± standard error.

Results

Stages of embryonic mortality

We analysed 694 clutches laid by 207 leatherback females during the five nesting seasons. In total, 39,690 eggs were laid of which 19,701 failed to hatch. Of the failed eggs, 11,728 (57.2%) failed at stage 0 and 2,100 (10.7%), 694 (3.5%) and 5,629 (28.6%) eggs failed at stages 1, 2 and 3 respectively. Results of the ANOVA and Tukey's test indicated a significant difference between all mean log transformed percent mortality values for each field stage ($df = 3$, $F = 959.24$, $p<0.01$). Mean hatching success was 50.4%.

Clutch effects

Three females laid an eighth clutch and one female laid a ninth clutch. Due to small samples sizes, these were removed for analysis. Remigrant females laid significantly more clutches than new nesters (2.5±0.1 and 2.0±0.1 clutches respectively; $L = 10.98$, $p<0.01$). However, mean clutch size did not differ significantly between remigrant (58.8±0.61 eggs) and new (57.4±0.78 eggs) females ($L = 0.22$, $p = 0.64$). In addition, clutch position had no significant affect on hatching success within a clutch ($L<0.01$, $p = 0.99$). Although not significant, there was a general trend in the data for the mean hatching success rate to decrease with each successive clutch (Table 1). The mixed model that excluded clutch position as an effect was significantly better at explaining variance in the stage of embryonic death than the model that included clutch position ($L = 28.21$, $p<0.01$) so, clutch position also did not significantly affect stage of embryonic death.

Female effects

There was a significant relationship between maternal identity and the hatching success in each clutch. ($L = 56.03$, $p<0.01$). Reproductive status of the female also significantly affected hatching success ($L = 8.82$, $p<0.01$), with a mean hatching success rate for remigrants of 52.40±1.18% (127 females, 516 clutches) and 44.22±2.01% for new nesters (80 females, 178 clutches). The interclutch interval did not differ significantly between remigrant (9.60±0.08 days) and new (9.69±0.09 days) females ($L = 1.98$, $p = 0.16$). There was a significant increase in mean percent hatching success with an increase in the duration of the interclutch interval ($L = 10.54$, $p<0.01$, $R^2 = 0.04$).

Females' remigrant or new reproductive status did not significantly affect the stage at which embryos died ($L = 0.64$, $p = 0.89$), but the interclutch interval did ($L = 54.83$, $p<0.01$). Mixed model reconstruction to include stage of death as a subset showed that Stage 0 ($L = 4.68$, $p = 0.03$), Stage 1 ($L = 8.41$, $p<0.01$) and Stage 3 embryonic death ($L = 12.45$, $p<0.01$) were all significantly affected by the interclutch interval, while Stage 2 ($L = 3.71$, $p = 0.05$) was not (Figure 1). Embryonic mortality during stage 3 was highest when the interclutch interval was 7 days (56±15.04%) and lowest at 12 days (14.7±4.29%). The reverse occurred in the number of embryos dying during stage 0 with mortality lowest when the interclutch interval was 7 days (28.2±10.91%) and highest at 11 days (62.8±3.48%) before a decline in mortality when the interclutch interval was 12 days (51.67±5.45%). Embryonic mortality increased again at both stages 0 and 3 when the interclutch interval was 13 days (62±6.50% and 23±7.40% respectively) (Figure 1). Average mortality values during stages 1 and 2 appeared to remain relatively constant, although there was a rise in mortality at an interclutch interval of 12 days (24.80±4.09% and 8.80±2.46% respectively) (Figure 1).

Season effects

Season significantly affected hatching success ($L = 90.98$, $p<0.01$). Lowest hatching success was during the 2006/07 nesting season with a mean percent hatching success of 38.16±2.07%. The highest hatching success was during the 2007/08 season with a mean percentage of 64.61±1.6% (Table 2). The season also significantly affected the stage of embryonic death ($L = 105.51$, $p<0.01$) with stages 0, 1 and 3 being significantly affected

Table 1. Mean percent hatching success (± standard error) and number of clutches in relation to clutch chronological position.

	Clutch identity						
	Clutch 1	Clutch 2	Clutch 3	Clutch 4	Clutch 5	Clutch 6	Clutch 7
Mean percent hatching success	51.25±1.76	52.78±2.00	48.94±2.46	51.98±3.11	45.44±3.80	43.50±5.53	32.97±8.60
Number of clutches	244	181	115	75	43	23	9

doi:10.1371/journal.pone.0021038.t001

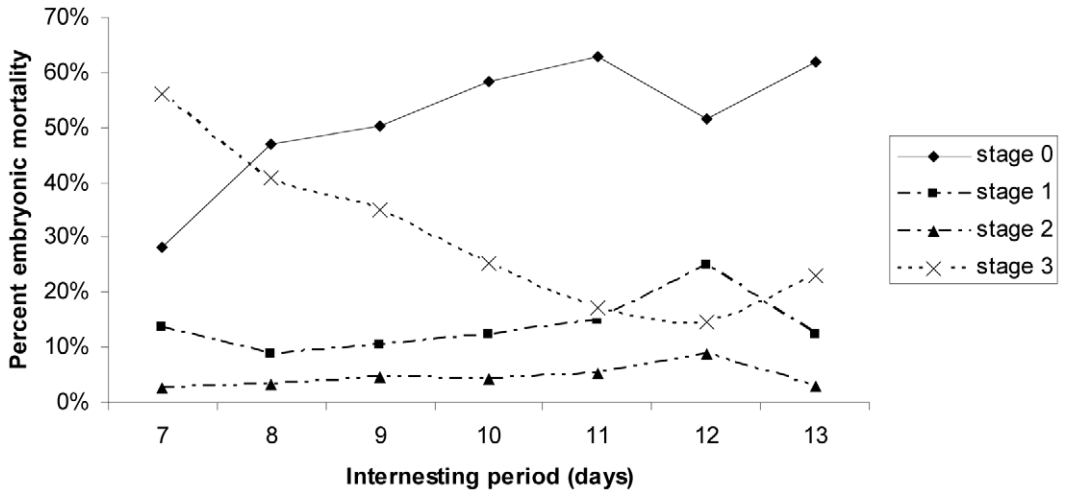

Figure 1. Relationship between the mean percentage of embryos dying at each field stage and interclutch interval (days).
doi:10.1371/journal.pone.0021038.g001

(L = 26.98, p<0.01; L = 43.84, p<0.01 and L = 16.01, p<0.01 respectively), whereas stage 2 was not (L = 8.00, p = 0.09). Across all seasons, mean percent embryonic mortality was consistently higher at stage 0 (56.2±1.0%) than all other stages (Figure 2). This was then followed by stage 3 (26.4±1.0%), stage 1 (13.1±0.6%) and stage 2 (4.2±0.3%), (Figure 2).

The nesting week during a season had no significant effect on hatching success within a clutch (L<0.01, p = 9.99). In addition, the mixed model that excluded nesting week as an effect was significantly better at explaining variance in the stage of embryonic death than the model that included it (L = 173.17, p<0.01) so, nesting week did not significantly affect stage of embryonic death. Nesting week also had no significant affect on the duration of the interclutch interval (L<0.01, p<0.01). However the interclutch interval was significantly affected by the status of the female, with remigrant females arriving earlier in the season to nest than new nesters (df = 1, F = 47.28, p<0.01; figure 3). Across all 5 nesting seasons, mean arrival time was 6.5±0.3 weeks for remigrant females and 9.7±0.4 weeks for new nesters (figure 3).

Discussion

During the study period, remigrant females arrived earlier in the season to nest, produced more clutches and had an overall higher rate of hatching success than new nesters. Maternal identity, maternal reproductive experience, interclutch interval and nesting season significantly affected hatching success of clutches, whereas clutch position and time of nesting did not. This agrees with previous research that showed hatchling production varies between nesting seasons at PNMB [18]. However, it differs from the findings presented by Garrett et al. (2010) showing that inter-clutch variation exists in hatching success among females in St Croix, and that a decrease in hatching success occurs with nesting season progression [18]. This latter result may have been an artefact of including 3 wet seasons and 2 dry seasons in this analysis, reducing the impacts that dry El Nino years have on hatching success, which generally result in increased mortality toward the end of the season (Santidrián Tomillo et al. unpublished data). This theory is supported by the apparent trend in the data, which, although it is not significant, suggests that hatching success decreases as the season progresses and each successive clutch is laid. Using an equal number of wet and dry seasons in future studies may accurately reflect this and is something that needs to be taken into consideration when conducting further investigations.

There was also a significant relationship between nesting season and interclutch interval, and the stages when embryos died. Clutch position and time of nesting had no significant affect on the proportion of embryos dying at different stages in this study. This suggests that within a given season embryos of certain females tend to die at a specific stage of development. The concept of 'good' and 'poor' mothers suggested by the data of Bell et al. (2003) seems to be explained at least in part by this maternal effect on the distribution of embryonic death in a clutch.

Some leatherback females consistently produce nests with higher hatching success rates than others [10]. The majority of embryonic death between the 2004/05 and 2008/09 nesting seasons occurred at field stage 0. This is also consistent with Bell et al. (2003) that most embryonic death in leatherback nests occurs primarily during early stages of development prior to and at Miller's (1985) stage 6. This pattern of embryonic mortality is contrary to that observed in St. Croix where most embryonic

Table 2. Mean percent hatching success (± standard error) and number of clutches per season.

	Season				
	2004/05	**2005/06**	**2006/07**	**2007/08**	**2008/09**
Mean percent hatching success	38.83±2.43	55.65±2.00	38.16±2.07	64.61±1.60	49.64±2.68
Number of clutches	128	171	129	158	108

doi:10.1371/journal.pone.0021038.t002

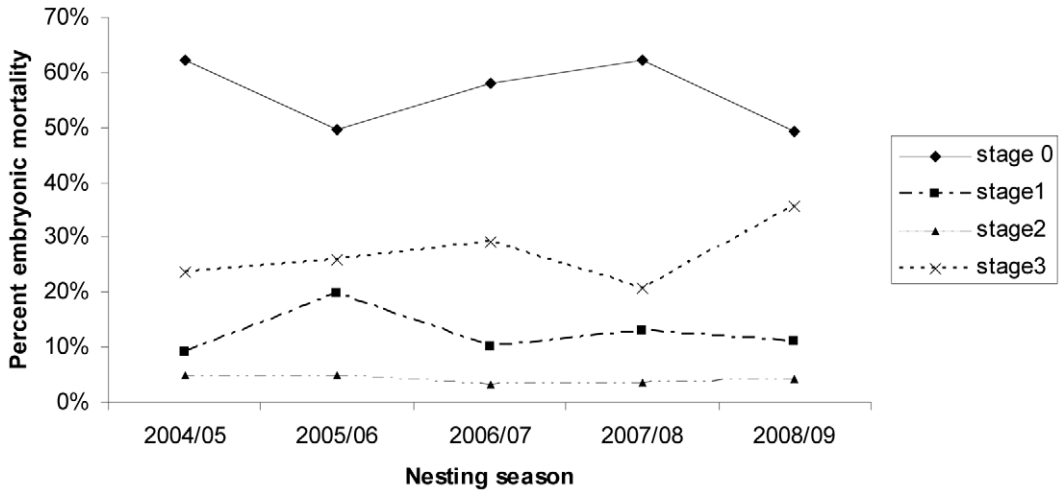

Figure 2. Relationship between the mean percentage of embryos dying at each field stage and nesting season (2004/05–2008/09).
doi:10.1371/journal.pone.0021038.g002

death occurs during late stages of development [16]. Regional variation in rates of hatching success of leatherbacks occurs. Investigations on leatherback nesting beaches include Tongaland, South Africa, 76% [43], Suriname, 50% [44], Rantau Abang, Malaysia, <56.4% [45], and Tortuguero, Costa Rica, 53.2% [13]. Variations in the distances travelled by females between breeding and feeding locations also exist in both the Atlantic and Pacific Oceans [1,2,3]. Distances and duration of nesting migrations can affect the health of mothers, thus causing variations in the amount of resources available for egg allocation and individual fecundity [46].

In turtles, maternal reproductive investment is directly represented by the allocation of energy and nutrients because there is no post-hatching parental care [33]. During reproduction, females produce amniotic eggs that consist of yolk and albumin encased in a proteinaceous shell. The proteins produced in the mother's oviduct largely dictate the internal incubation environment of the egg [47,48]. Reptiles are known to produce low molecular weight proteins in the oviduct that affect embryonic development [49]. In addition to these maternal influences on the embryo, the embryo is also capable of producing factors that affect oviducal activities [49]. Growth factors secreted by American alligators (*Alligator mississippiensis*) are present in the oviducal secretions of gravid females and become incorporated into egg albumin and affect embryonic development [47,49,50]. Preliminary work on turtles has identified similar growth factors in slider turtle (*Trachemys scripta*) plasma [51]. Maternal effects have been used to describe differences in the growth rate and righting response of snapping turtle (*Chelydra serpentine*) hatchlings [39,52]. It is therefore plausible that female turtles directly influence the development of their embryos inside the oviduct and possibly after oviposition.

In addition to water and solids, mothers supply the albumin of eggs with factors necessary for development [20,53]. Insulin-like growth factors I and II have been identified as products that are secreted by the reptilian reproductive tract [47,51]. These factors influence development in a multitude of different species [54,55]. Binding proteins associated with IGFs (IGFBP) are genetically induced by hypoxia [56,57,58]. Embryonic metabolism is believed to arrest in response to hypoxia in turtle oviducts [37,59,60, 61,62,63], perhaps through the activity of IGFBP. Although this remains to be investigated, it may be plausible that the activity of growth factors present in the egg albumin is affected by

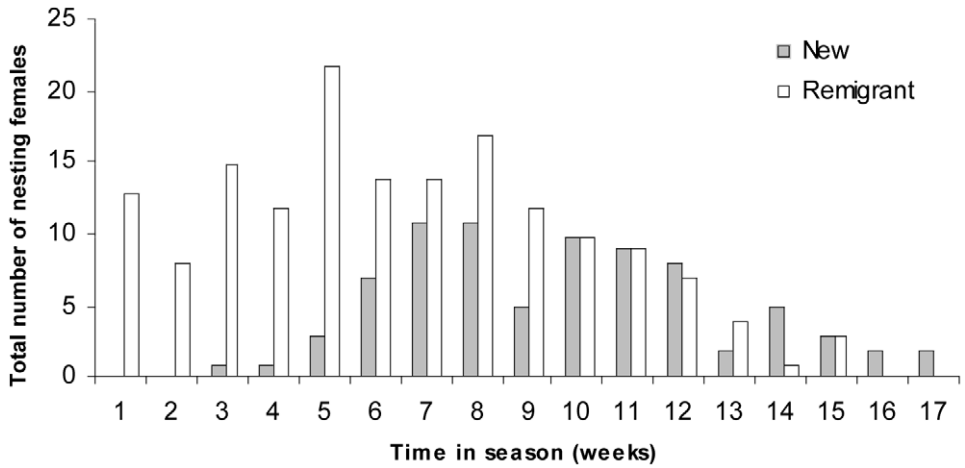

Figure 3. Total number of new and remigrant females nesting each week between 2004/05 and 2008/09.
doi:10.1371/journal.pone.0021038.g003

environmental variables such as oxygen both inside the female and the nest. Embryos of some females may be more developmentally sensitive to environmental conditions than others [30]. Environmental variability driven by the El Niño Southern Oscillation affects hatching and emergence success of leatherback turtles in Costa Rica (Santidrián Tomillo et al. unpublished data). Evaluating maternal environmental effects in an ecological context has demonstrated that mothers may provide phenotypic adaptation to local environmental conditions [64,65,66].

There is no significant difference between the interclutch intervals of remigrant and new females. This is consistent with the result that the interclutch interval does not change as the season progresses, regardless of the different peak nesting periods for both new and remigrant females. New females have lower rates of hatching success than remigrant females, but female status does not affect the stage at which embryos die. This suggests that it is the duration of exposure to the maternal oviducal environment that is affecting the stage of embryonic death rather than the experience of the female. Embryonic death at field stage 3 steadily declines as the interclutch interval gets longer and approaches 12 days. Although it remains to be tested, perhaps additional time spent in the hypoxic environment of the oviduct conditions the embryos in some way to later withstand hypoxia in the nest. The maternal provisioning of glycogen metabolites to her embryos can affect their ability to survive low oxygen conditions in *C. elegans* [66]. Perhaps increased allocation of glycogen, or other oviducal factors, by some females *in utero* prevents embryonic mortality when oxygen tensions decrease in the nest during the final stages of incubation. This may explain why late stage embryonic mortality and hatching success is not significantly affected by hypoxia at PMNB [30,31]. Changes in oxygen and carbon dioxide partial pressures occur during the incubation period in response to the collective development of embryos [30,31,67]. Hypoxia and hypercapnia occur during the final stages of incubation between days 50 and 60 with declines in oxygen tensions and increases in carbon dioxide levels occurring from day 35 at PNMB [30]. The second half of development is the period when embryonic growth and development is most sensitive to changes in respiratory gas concentrations [68].

Olive ridley turtles (*Lepidochelys olivacea*) naturally have extended interclutch intervals of up to 63 days during which, embryos are maintained in a state of arrested development and subsequently resume development after oviposition [38]. This is likely a reproductive strategy this species employs to enable mass arribada nesting [69]. Delayed nesting and extended periods of egg retention have also been reported in various freshwater turtle species including *Deirochelys reticularia* (chicken turtle), *Kinosternon subrunrum* (eastern mud turtle) and *Trachemys scripta* (red-eared slider) [70,71]. It would be interesting for future research to identify how embryos are maintained in this arrested state for such long periods and how it might affect subsequent embryonic development.

In conclusion, in addition to seasonal effects on hatching success in leatherback turtles, some females are better mothers than others. The study of how intrinsic factors such as maternal effects impact upon sea turtle embryonic death is a new field and at this stage we can only speculate mechanisms that may be actively affecting embryonic mortality. We hypothesize that mothers may be affecting the development of their young by producing factors that become incorporated in the internal environment of the egg and infer different levels of developmental sensitivity to environmental conditions. By identifying the causes of embryonic death in the leatherback turtle, we can use this information as a baseline understanding of embryonic mortality in not just other chelonian species and reptiles in general, but also other egg laying animals. It may also allow strategies to be put into place that increase hatching success and production in this critically endangered species.

Acknowledgments

We thank field assistants and Earthwatch volunteers for help on data collection during the five field seasons, especially G. Blanco, T. Dornfeld and J. Suss. We are grateful to park rangers and volunteers for their contribution to the research projects and conservation efforts at PNMB. Thank you also to Christopher Johnstone, Andreas Svensson and John Morrongiello for their statistical advice.

Author Contributions

Conceived and designed the experiments: AR PST JS FP RR. Performed the experiments: AR PST JS FP RR. Analyzed the data: AR PST JS FP RR. Wrote the paper: AR PST JS FP RR.

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Chapter Four

Limited oxygen availability *in utero* may constrain the evolution of live-birth in reptiles

Anthony R. Rafferty[1], Roger G. Evans[2], T. Franciscus Scheelings[3] & Richard D. Reina[4]

[1]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

[2]Department of Physiology, Monash University, Melbourne, Victoria, Australia

[3] Australian Wildlife Health Centre, Healesville Sanctuary, Healesville, Victoria, Australia

Accepted for publication under the same name in American Naturalist

Accepted 1st September 2012

Declaration for Thesis Chapter Four

Monash University

Declaration by candidate

In the case of Chapter four, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation	79%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
T. Franciscus Scheelings	Data collection and manuscript editing	7%
Roger Evans	Design, data collection, statistical analysis and manuscript editing	7%
Richard Reina	Supervision, data collection, manuscript editing and direction	7%

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Monash University, School of Biological Science, Clayton campus

	Signature	**Date**
T. Franciscus Scheelings		09/05/2012
Roger Evans		09/05/2012
Richard Reina		

Green turtle (*Chelonia mydas*) eggs visible in the nest cavity during oviposition
Photo taken by T. Franciscus Scheelings

Abstract

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

4.1 Introduction

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

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

The onset of preovipositional arrest, or the occurrence of developmental deformity in its absence, suggests that there is a factor that limits development in the oviduct that thereby constrains the evolution of viviparity in extant oviparous reptiles. Restricted gas and/or water availability in the absence of oviducal structures that permit their exchange are plausible restraints likely to limit development (Guillette 1989; Guillette 1993). However, restricted oviducal water availability is not a limiting factor during *Sceloporus* lizard embryonic growth (Andrews 2004; Mathies and Andrews 1995). In addition, although reptilian eggs typically absorb water during growth, hydric conditions have a relatively minor impact on development inside rigid shelled eggs when compared to flexible shelled eggs (Packard 1991; Packard and Packard 1988). Furthermore, the onset of preovipositional arrest in rigid and flexible shelled chelonian eggs occurs at the same development stage despite eggshell type (Ewert 1985), suggesting that restricted water availability is not the primary limiting factor for chelonians either.

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

In order to establish whether restricted O_2 availability in the reptilian oviducal environment limits embryonic development, we investigated whether hypoxia affects active embryonic development, determined the oviducal oxygen partial pressure (PO_2) experienced by reptilian embryos and assessed the rate of O_2 diffusion in reptilian oviducal secretions. Turtles are ideal study animals to investigate our proposed role for hypoxia as a developmental regulator because they are the only reptile group in which all species undergo a period of preovipositional developmental arrest (Ewert 1985; Miller 1985). We used three species of freshwater turtle, in addition to one species of sea turtle, to show that restricted oviducal O_2 availability limits embryonic development.

4.2 Methods

4.2.1 Study animals

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

4.2.2 Effects of hypoxia on eggs in pre-ovipositional arrest

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

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

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

The hypoxic treatments were created using airtight Perspex containers (Resi-Plex Plastics, Vic, Australia) measuring 26 cm x 15.75 cm x 11 cm, with an inflow and outflow valve at either end. Pure nitrogen gas (BOC Gases, Australia) was pumped at a constant rate into each chamber via the gas inlet, beginning approximately 3 minutes prior to introduction of the first egg, until valve closure. The gas was humidified by first bubbling through distilled water in a separate airtight flask. The lid of each chamber was lifted briefly and replaced after each egg was added. Eggs were positioned on wire mesh above 20 mL of distilled water in each chamber. Eggs were added to each chamber as they were laid, for up to 10 minutes after the first egg was introduced. At this time, the chamber was sealed and the nitrogen gas was allowed to flow through the chamber for an additional three minutes before both valves were closed, sealing the chamber. The O_2 tension of the gas escaping the chamber via the outlet valve was measured continuously during this 3-minute interval using an O_2 sensor (Analytical Industries Incorporated, USA) attached to a data

collection unit (Pasco, USA) calibrated using atmospheric air. The final O_2 tension of each chamber was noted when the valves were closed and never exceeded 7.6 mmHg (~1% O_2).

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

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

4.2.3 O_2 partial pressure (PO_2) in the oviducal environment

Prior to measurement of O_2 partial pressure (PO_2) in the oviduct, gravid freshwater turtles were restrained in individual cotton bags and placed in 32°C incubators to increase body temperature for at least one hour. Each turtle was then removed from the bag, weighed and manually restrained in a right lateral recumbent position with the head distal to the anesthetist. Anesthesia was induced by IV injection of alfaxalone (Alfaxan CD RTU®, 10 mg/ml alfaxalone) at a dose 8

mg/kg via the jugular vein. Gravid *C. mydas* were obtained from the nesting beach and manually restrained prior to the procedure. Internal temperature was recorded in each female using a digital thermometer inserted into the cloaca.

PO_2 in the oviduct (mmHg) was measured using an LAS-1 fiberoptic probe (1 mm in diameter with sampling area 1 mm X 1 mm; Oxford-Optronix, Oxford, United Kingdom) attached to an OxyLite fluorescence lifetime oximeter set to record PO_2 at one-second intervals (Oxford-Optronix, United Kingdom). The OxyLite device was interfaced with a data acquisition system (Powerlab, AD Instruments, Sydney, Australia) connected to a laptop computer. Temperature compensation was performed manually, by setting the OxyLite temperature value to the internal temperature recorded for each turtle. The fiberoptic probe was threaded through an endoscope so that the tip of the probe was protruding approximately 1.5 mm from the distal aperture of the endoscope. Both the endoscope and the probe were directed through the cloacal opening of each turtle and into one of the oviducts. Images from the endoscope's video camera were displayed on a television monitor, which allowed confirmation that the O_2 measurement probe was inside the oviduct. A reading was accepted if the recorded PO_2 remained within $\pm$ 0.5 mmHg for at least ten seconds. Measurements were attempted in both the right and left oviduct of each turtle.

4.2.4 O_2 diffusion in oviducal secretions

Collection of oviducal fluid: Oviducal secretion samples were collected from 16 ovipositing green turtles. During oviposition, a 22 cm x 12.5 cm Ziploc bag was placed under the cloaca of each female after approximately 10 eggs had been laid into the nest chamber. Subsequent eggs were laid with their adhering oviducal secretions directly into the bag. Eggs were removed by hand as they were deposited so that only the secretion remained in the bag. This continued until approximately 50 eggs had been laid into the bag. Secretion samples were then transferred to individual 5 ml vials, sealed and refrigerated at 5 °C for up to 48 hours until samples had been obtained from all females.

Measurement of O_2 *diffusion:* One mL of oviducal secretion from each of the 16 females was pooled and the mixed secretion sample was re-distributed evenly between eight 1.5-mL eppendorf tubes. Five 1.5-mL eppendorf tubes were also filled with 154 mM NaCl (saline) and acted as a control for this experiment. O_2 diffusion was measured in each of these eight secretion samples and five saline samples using a polarised Clark electrode (50 μm tip, Unisense, Århus, Denmark). The

electrode was calibrated prior to use in a calibration chamber with pure O_2 (760 mmHg O_2) and pure nitrogen (0 mmHg O_2). Once calibrated, the probe was positioned using micromanipulators, 2 mm below the surface of each secretion or saline sample. The probe was left standing in the sample for approximately 5 minutes until PO_2 stabilised. Pure O_2 gas was then blown across the surface of the sample at a constant rate of 1 L min^{-1} from an outlet (a glass Pasteur pipette) positioned at a 30º angle 15 mm from the edge of the eppendorf tube. PO_2 at the probe tip was sampled at one-second intervals for a 10-minute period from the time the O_2 gas began blowing across the sample surface.

4.2.5 Data analysis

The proportion of eggs to form white spots in the normoxic and hypoxic treatments were compared using Pearson's Chi-squared test. The proportion of eggs to form white spots was calculated from the number of eggs remaining in each treatment group after some eggs had been dissected to identify their developmental stage. The time elapsed (days) from oviposition to white spot formation was recorded and also from the time that each hypoxic treatment finished until white spot formation. An ANCOVA was used to identify whether the duration of hypoxia affected the period between oviposition and white spot formation. These data were also subjected to analysis of variance and Tukey's HSD tests to determine whether the duration of hypoxia affected the length of time between the end of the hypoxic treatment and white spot formation.

Oviducal PO_2 measurements were analysed using LabChart 7 (AD Instruments, Sydney, Australia). For each oviduct, five consecutive values within the 10-second plateau period were averaged to obtain a single O_2 value for that oviduct. Paired t-tests were used to compare the left and right oviduct PO_2 values within each species.

PO_2 values measured by the Clark electrode in oviducal fluid and saline were recorded at 1 s intervals (Universal Acquisition, University of Auckland). These values were binned into one-minute means for each fluid type for subsequent analysis. A linear mixed effect model was run in R statistical software (R core development team 2011; http://www.r-project.org/) using the 'nlme' package to identify whether the rate of diffusion differed between the secretion and saline samples. The rate of diffusion in each sample was treated as the response variable, sample type (secretion or saline) was the fixed effect and sample number was the random effect. For all

analyses two tailed $P \leq 0.05$ was considered statistically significant and data presented are mean ± standard error.

4.3 Results

4.3.1 Effects of hypoxia on eggs in pre-ovipositional arrest

In total, 74 eggs from twelve *C. oblonga*, 68 eggs from nine *C. longicollis*, 76 eggs from six *E. macquarii* and 82 eggs from two *C. mydas* females were used in the study. The distribution of eggs between treatment groups for each species is shown in Table 1.

Preovipositional developmental arrest was prolonged in a hypoxic environment. No eggs developed white spots (the first visual sign that preovipositional arrest has broken and the egg is developing) during the 3 day, 6 day or 9 day hypoxic treatments (Figure 1). This resulted in a significant increase in the time from oviposition to formation of a white spot, as the period of hypoxic incubation increased from 0 to 3, 6 and 9 days for *C. oblonga* ($Df = 23$, $F = 16.46$, $P = 0.001$, slope = 1.89), *C. longicollis* ($Df = 36$, $F = 30.85$, $P < 0.001$, slope = 1.14), *E. macquarii* ($Df = 54$, $F = 604.5$, $P < 0.001$, slope = 1.13) and *C. mydas* ($Df = 63$, $F = 4366$, $P < 0.001$, slope = 1.53) (Table 1). In the normoxic control, *C. mydas* eggs took an average 1.3 ± 0.1 days to form white spots, *E. macquarii* eggs took 2.1 ± 0.2 days, and *C. longicollis* and *C. oblonga* eggs took 2.6 ± 0.8 and 11.5 ± 1.7 days respectively.

All freshwater and marine turtle eggs dissected at oviposition and after 3, 6 and 9 days in hypoxia were at Yntema's (1968) stage 0 and Miller's (1985) stage 6 of development respectively, indicating that developmental arrest had continued during the treatment. Successful recommencement of development and the formation of white spots at the conclusion of each treatment was seen when eggs were subsequently exposed to normoxia. However, the proportion of eggs that successfully recommenced development differed significantly between treatments for both *C. oblonga* ($Df = 3$, $X^2 = 15.14$, $P < 0.001$) and *C. mydas* ($Df = 3$, $X^2 = 20.60$, $P < 0.001$), with the number of eggs surviving the hypoxia generally declining with increased duration in hypoxia. The proportion of eggs dying increased from 21.1% in the normoxic control to 80% in the 9-day hypoxic treatment for *C. oblonga* and from 0% to 26.7% between the normoxic and 9-day hypoxic treatment respectively, for *C. mydas* (Table 1). No significant difference was observed in the proportion of eggs dying in the normoxic or hypoxic treatments for *C. longicollis* ($Df = 3$, $X^2 =$

1.68, P = 0.64) or *E. macquarii* (Df = 3, X^2 = 7.17, P = 0.07). However, a trend towards an increase in the proportion of eggs dying with increased duration in hypoxia was observed in both cases. An increase from 12.5% mortality in the normoxic control to 33.3% in the 9-day hypoxic treatment occurred for *C. longicollis* and from 0% to 27.7% for *C. mydas* (Table 1).

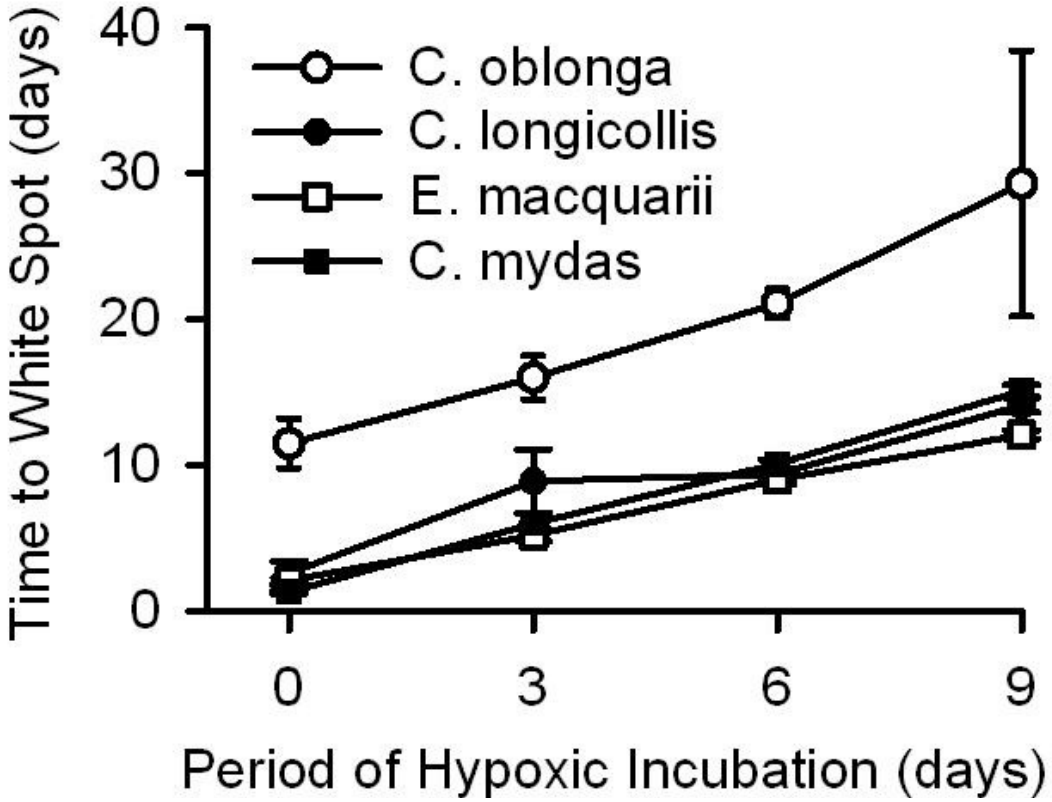

Figure 4.1. Mean time (days) from oviposition to white spot formation of eggs in normoxic (0 days) and hypoxic (3 days, 6 days and 9 days) treatments ± standard error (SEM).

The period of time it took to resume development and form a white spot after each hypoxic treatment finished generally exceeded the time actually spent in hypoxia. In addition, the time to white spot formation generally became longer with increased duration in hypoxia, relative to the normoxic control. There was a significant increase in the number of days to formation of white spot as the period of hypoxia progressed from 0 days in the normoxic control to 3 days, 6 days and 9 days for *C. mydas* (Df = 3, F = 176.02, P < 0.001). A significant difference in the time to white spot development was also identified between the eggs in the normoxic control and the 3-day, 6-day and 9-day hypoxic treatments for *E. macquarii* (Df = 3, F = 3.02, P = 0.04), although a post-hoc Tukey's HSD test did not identify any specific treatment as significantly different from another. The duration of hypoxia did not significantly affect the time from exposure to

normoxia to white spot formation of eggs of *C. longicollis* (Df = 3, F = 1.32, P = 0.29) or *C. oblonga* (Df = 3, F = 1.19, P = 0.34) (Table 1).

4.3.2 O_2 partial pressure (PO_2) in the oviducal environment

PO_2 was measured in both left and right oviducts for four *C. oblonga*, seven *C. longicollis* and three *E. Macquarii* turtles, in addition to 11 single oviduct readings (three right and eight left) for *C. mydas* turtles. Three single right oviduct measurements and one single left oviduct measurement were obtained from an additional four *C. oblonga*. Paired t-tests were used to compare the left and right oviduct PO_2 values within each species (except the marine turtles in which only one oviduct was sampled in each female). Results indicated that the PO_2 measured in the left oviduct did not differ significantly from that in the right oviduct in *C. oblonga* (Df = 3, t = 2.50, P = 0.09), *C. longicollis* (Df = 6, t = 0.98, P = 0.36) or *E. macquarii* (Df = 2, t = 0.39, P = 0.73) so the 4 single oviduct readings for *C. oblonga* were included in the sample used for calculation of the between-animal mean value for that species. Mean oviducal PO_2 was 5.9 ± 2.5 mmHg in *C. oblonga* (n = 8); 1.6 ± 1.2 mmHg in *C. Longicollis* (n = 7); 5.3 ± 2.1 mmHg in *E. macquarii* (n = 3); and 2.9 ± 1.4 mmHg in *C. mydas* (n = 11) (Figure 2).

4.3.3 O_2 diffusion in oviducal secretions

The mean PO_2 measured at the beginning of the experiment was significantly lower in oviducal secretions (84.6 ± 3.3 mmHg; range 72.4 – 99.8 mmHg) than in saline (126.3 ± 12.6 mmHg; range 86.6 – 160.4 mmHg) (Df = 1, F = 15.6, P = 0.002) (Figure 3). Exposure of the sample to a stream of 100% O_2 resulted in a progressive increase in PO_2 in all samples. However, the rate at which PO_2 increased over the 10 minutes of exposure to 100% O_2 was significantly greater for saline (14.2 ± 2.1 mmHg/min) than for the oviducal secretion (1.9 ± 0.6 mmHg/min) (Df = 1, F = 64.3, P = 0.001). Furthermore, the mean PO_2 after 10 minutes of exposure to 100% O_2 was significantly lower in the oviducal secretion samples (103.5 ± 5.8 mmHg; range 87.1 – 134.0 mmHg) than the final measurement recorded in the saline (268.3 ± 30.2 mmHg; range 216.4 – 380.4 mmHg) (Df = 1, F = 45.4 P < 0.001) (Figure 3).

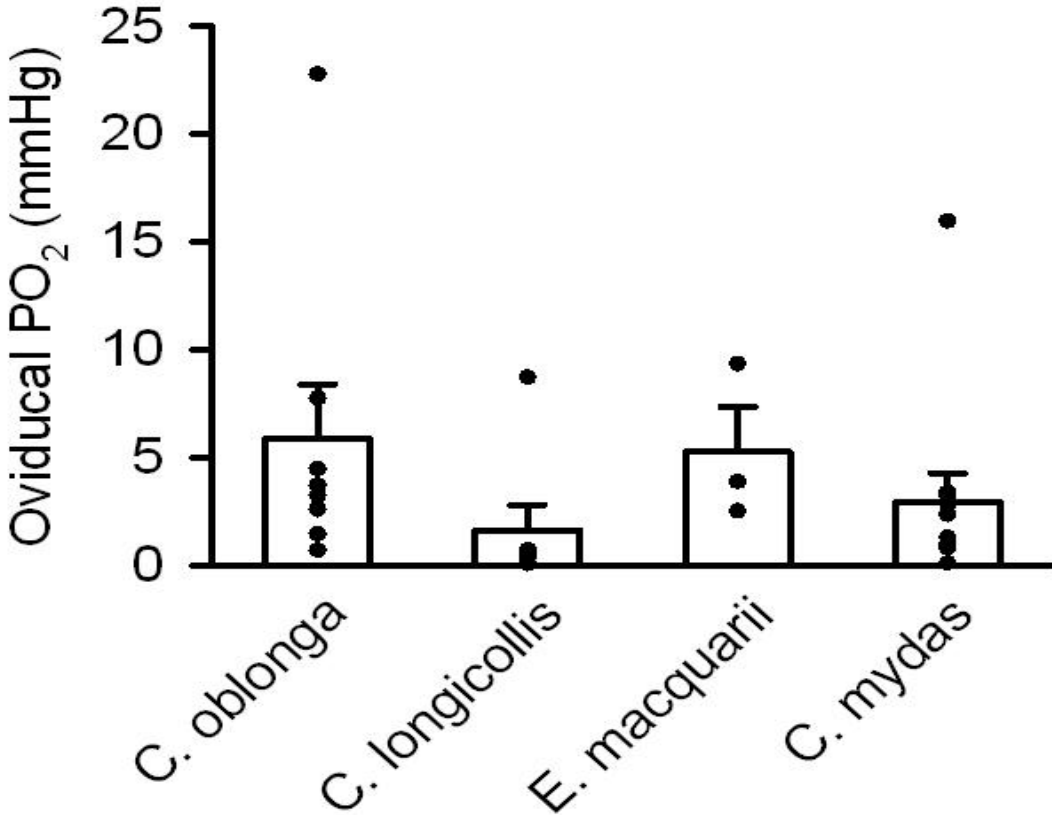

Figure 4.2. Partial pressure of oxygen (PO_2) in gravid oviducts of *Chelodina oblonga* (n = 8), *Chelodina longicollis* (n = 7), *Emydura macquarii* (n = 3) and *Chelonia mydas* (n = 11) ± standard error (SEM).

4.4 Discussion

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

Table 4.1. Effects of hypoxia on egg development

	Control (normoxia)	Hypoxia 3 day	Hypoxia 6 day	Hypoxia 9 day
C. oblonga				
Number of eggs in treatment (*n*)	24	15	15	20
Number of eggs dissected after treatment[1]	5	6	5	5
Number of remaining eggs to form white spot[2]	15 (78.9%)	4 (44.4%)	2 (20.0%)	3 (20.0%)
Number of eggs failing to begin development[3]	4 (21.1%)	5 (55.6%)	8 (80.0%)	12 (80.0%)
Mean days to white spot after treatment (± SEM)	11.5 ± 1.7 days	13.0 ± 1.5 days	15.0 ± 1.0 days	20.3 ± 9.1 days
C. longicollis				
Number of eggs in treatment (*n*)	13	17	18	20
Number of eggs dissected after treatment[1]	5	5	5	5
Number of remaining eggs to form white spot[2]	7 (87.5%)	10 (83.3%)	10 (76.9%)	10 (66.7%)
Number of eggs failing to begin development[3]	1 (12.5%)	2 (16.7%)	3 (23.1%)	5 (33.3%)
Mean time to white spot after treatment (± SEM)	2.6 ± 0.8 days	5.9 ± 2.2 days	3.4 ± 0.4 days	5.1 ± 0.5 days
E. macquarii				
Number of eggs in treatment (*n*)	19	22	18	17
Number of eggs dissected after treatment[1]	4	4	3	2
Number of remaining eggs to form white spot[2]	15 (100%)	17 (94.4%)	14 (93.3%)	11 (73.3%)
Number of eggs failing to begin development[3]	0 (0%)	1 (5.6%)	1 (6.7%)	4 (27.7%)
Mean time to white spot after treatment (± SEM)	2.1 ± 0.2 days	2.2 ± 0.4 days	3.0 ± 0.3 days	3.1 ± 0.3 days
C. mydas				
Number of eggs in treatment (*n*)	35	16	15	16
Number of eggs dissected after treatment[1]	3	1	1	1
Number of remaining eggs to form white spot[2]	32 (100%)	14 (93.3%)	7 (50.0%)	11 (73.3%)
Number of eggs failing to begin development[3]	0 (0%)	1 (6.7%)	7 (50.0%)	4 (26.7%)
Mean time to white spot after treatment (± SEM)	1.3 ± 0.1 days	3.0 ± 0.0 days	4.1 ± 0.3 days	6.1 ± 0.4 days

[1]Total number of eggs dissected in each treatment to identify the stage of embryonic development

[2]Total number of eggs to form white spots in each treatment, not including the eggs that were dissected to identify developmental stage

[3]Total number of eggs that failed to develop a white spot and were considered dead in each treatment

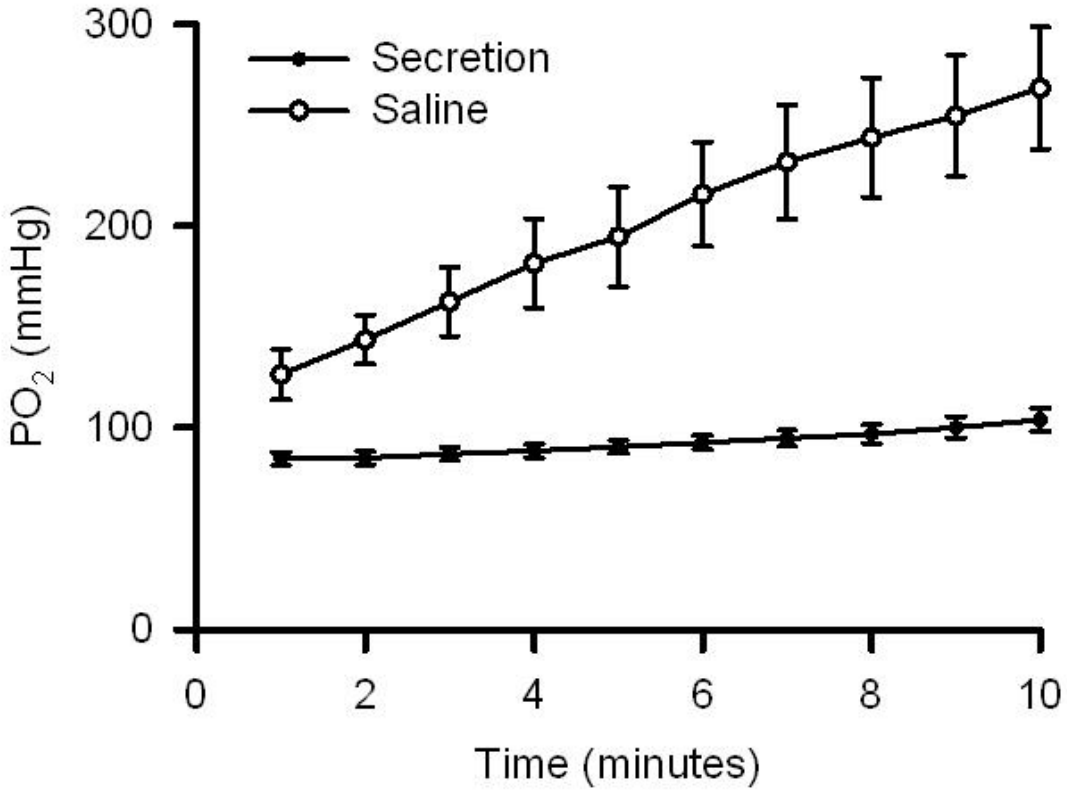

Figure 4.3. Comparison of rates of oxygen diffusion over 10 minutes in saline (n = 5) and oviducal secretions (n = 8) of the green turtle, *Chelonia mydas*. Each point represents the between sample mean ± standard error (SEM) of 1 minute averages.

Marine and freshwater turtle embryos typically arrest during early development stages; stage 6 of 31 for marine embryos (Miller 1985) and stage 0 of 26 for freshwater embryos (Ewert 1985), whereas lizard embryos typically arrest at around stage 30 of a 40 stage developmental chronology (Andrews and Mathies 2000). Mean PO_2 in turtle oviducts is substantially lower than estimates in sceloporine lizards, which is suggested to exceed 40 mmHg (Parker and Andrews 2006). It is therefore plausible that turtle embryos become arrested during earlier stages than lizard embryos because lower oviducal O_2 tensions limit development earlier in the developmental schedule (Andrews and Mathies 2000; Parker and Andrews 2006). Therefore, we hypothesise that the onset of preovipositional arrests occurs when embryonic oxygen demand exceeds its availability in the oviduct. However, further investigations are needed to quantify embryonic oxygen demand *in utero*, and the simultaneous availability of oxygen availability in the oviduct, to confirm whether this is the case.

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

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

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

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

Our findings indicate that extended periods of preovipositional arrest in response to hypoxia (simulating extended oviducal egg retention) significantly reduce survival of *C. oblonga* and *C. Mydas* embryos, at least under our experimental conditions. Developmental deformities and death of turtle embryos has been previously linked to periods of extended egg retention and prolonged preovipositional arrest (Ewert 1985). Embryonic death of sceloporine lizard embryos also increases during periods of experimentally induced hypoxia (Parker and Andrews 2006). Therefore, it is possible that extended periods in hypoxia can negatively affect embryonic survival, thus selecting for shorter egg retention intervals that ultimately prevent the evolution of viviparity (Rafferty et al. 2011). Extended oviducal egg retention decreases post-ovipositional embryonic survival early in development in naturally nesting leatherback turtles (Rafferty et al. 2011). However, it is also possible that increased mortality in laboratory tests is due to artificial manipulation of the incubation environment because no significant difference was observed in the proportion of eggs dying in the normoxic or hypoxic treatments for *C. longicollis* or *E. macquarii* even though they were incubated under the same conditions as the two other species.

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

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

4.5 Acknowledgements

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

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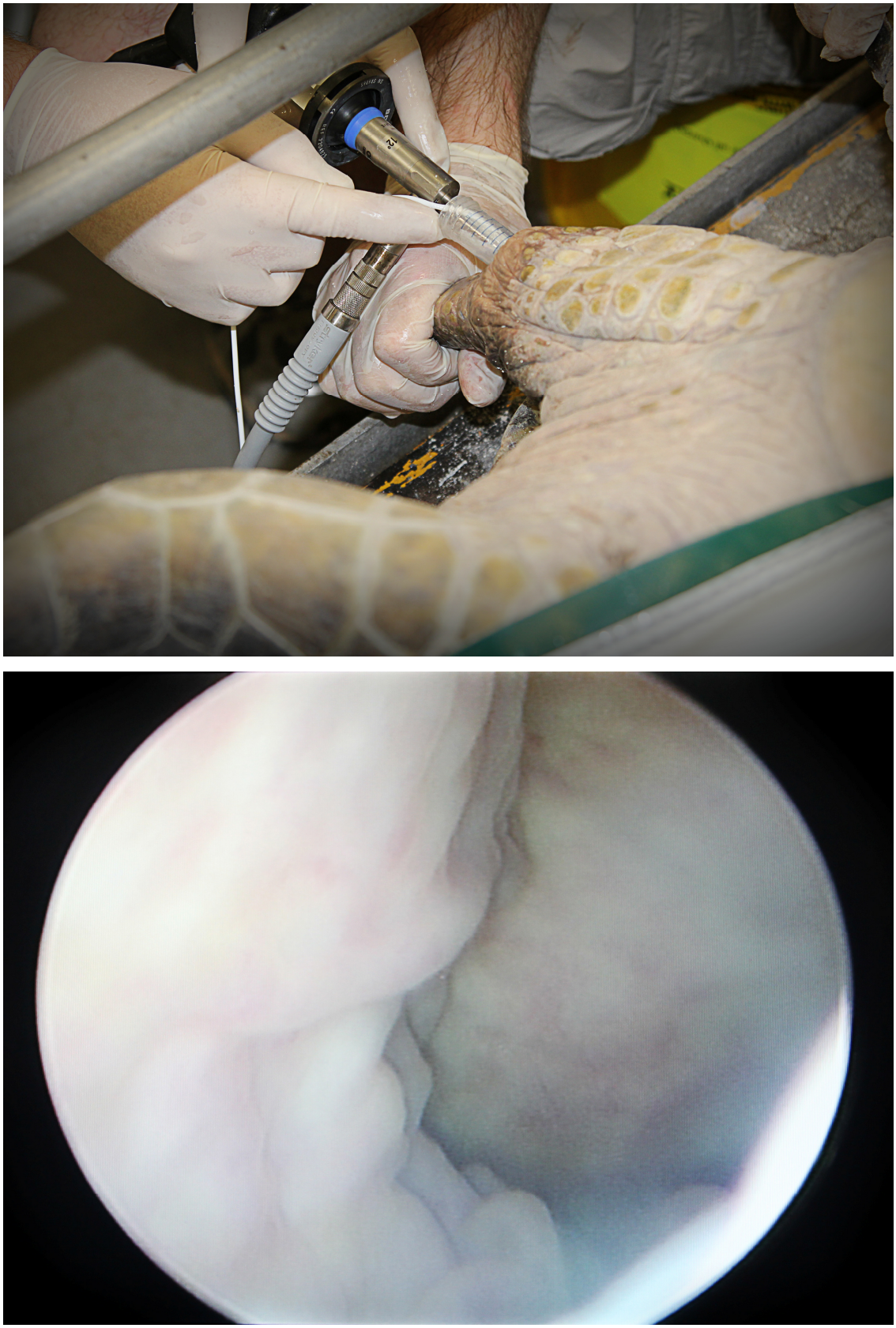

Top: Measuring the oviducal oxygen tension in a gravid green turtle (*Chelonia mydas*)
Bottom: An endoscopic image of the green turtle (*Chelonia mydas*) oviduct
Photos taken by T. Franciscus Scheelings

Top: A green turtle (*Chelonia mydas*) in the process of laying her eggs
Bottom: Collection of green turtle (*Chelonia mydas*) eggs and oviducal fluid
Photos taken by T. Franciscus Scheelings

Chapter Five

The influence of temperature on arrested embryonic development in turtles

Anthony R. Rafferty[1] & Richard D. Reina[1]

[1]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

In review under the same name in Journal of Experimental Biology

Submitted 25th May 2012

Declaration for Thesis Chapter Five

Monash University

Declaration by candidate

In the case of Chapter five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation	90%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Richard Reina	Supervision, manuscript editing and direction	10%

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Monash University, School of Biological Science, Clayton campus

	Signature	**Date**
Richard Reina		

Green turtle (*Chelonia mydas*) hatchling just after emergence from the shell
Photo taken by Rohan Clarke

Abstract

Arrested embryonic development is an important reproductive strategy in the large range of egg laying animals that provide no parental care after oviposition, because it gives their eggs some capacity to respond to changing environmental conditions during embryonic development. We investigated whether incubation temperature influenced arrested embryonic development in eggs of the green sea turtle, *Chelonia mydas*, and three species of freshwater turtle; the western oblong turtle, *Chelodina oblonga*, the eastern longneck turtle, *Chelodina longicollis*, and the Murray River turtle, *Emydura macquarii*, by incubating eggs of each species at three different temperatures and monitoring embryonic development. Our results demonstrated that the breaking of pre-ovipositional arrest after eggs are laid occurred independently of temperature in all four species. However, temperature influenced subsequent development beyond this point and at 22ºC, 64% of *E. macquarii* embryos developed to stage 25 of a 26 stage developmental chronology for this species, but failed to hatch. Similarly, at 24ºC, 45% of *C. mydas* embryos failed to hatch and died at stage 30 of a 31 stage developmental chronology. Presumably, this was because embryos of both species entered a state of delayed hatching, awaiting a necessary stimulus to trigger pipping that never arose at constant temperature. These findings represent the first evidence that sea turtles possibly retain remnant traces of an ability to delay hatching that they once possessed, or perhaps that they may be pre-adapted to evolve delayed hatching in the future.

5.1 Introduction

Incubation temperature largely dictates the rate of embryonic growth and development in reptiles in addition to influencing hatchling phenotype, including sex (Deeming & Ferguson 1991; Crews *et al.* 1994). Fluctuations in temperature during incubation can change the developmental rate of embryos and alter incubation period (Georges *et al.* 2004). In some instances, incubation duration can be extended significantly if embryos enter a state of developmental arrest in response to temperature, which involves the down-regulation or cessation of cell division and metabolic activity (Ewert 1991). Developmental arrest is a critically important reproductive strategy for many oviparous (egg-laying) amniotes because it allows some embryonic resilience to environmental conditions that do not favour successful development (Rafferty & Reina 2012). It also provides embryos the capacity to synchronise hatching with seasonal periods that benefit the resulting offspring (Ewert 1991).

Developmental arrest is predominantly observed in extant reptiles (squamates, crocodiles, turtles and the tuatara) and it generally occurs at least once during development inside the amniotic egg in reptile orders (Rafferty & Reina 2012). Pre-ovipositional arrest occurs while eggs are inside the oviduct and persists until after oviposition, regardless of the duration of egg retention. The degree of embryonic development that takes place prior to the onset of pre-ovipositional arrest varies largely among and even within different capable species. For example, the common chameleon, *Chamaeleo chamaeleon*, the tuatara, *Sphenodon punctatus*, and all marine and freshwater turtles arrest as gastrulae (Ewert 1985; Miller 1985; Moffat 1985; Miller 1997; Andrews *et al.* 2008), whereas the North American iguanids, *Urosaurus ornatus* and *Sceloporus undulatus*, arrest during the final stages of development if hatching is imminent and the eggs have not been laid (Radder *et al.* 1998; Andrews & Mathies 2000). Although temperature is unlikely to inhibit oviducal development (Ewert 1985; Rafferty & Reina 2012), it may dictate when development resumes after eggs have been laid (Booth 2002a).

Some reptile embryos enter a subsequent developmental arrest shortly after oviposition, termed embryonic diapause. Embryos from several chamaeleonid lizard lineages undergo a period of post-ovipositional embryonic diapause while they are still gastrulae (Ewert & Wilson 1996; Booth 2002b), whereas turtles generally become arrested just prior to somite and vitelline development (Ewert & Wilson 1996; Booth 2002a). It is thought that in warm temperate regions where oviposition is seen in autumn, embryonic diapause occurs in response to temperature changes (Ewert 1991; Ewert & Wilson 1996). Embryonic diapause occurs in healthy embryos that are in an environment that normally promotes active development and without an obligatory trigger to re-start development, embryos remain arrested and eventually die (Ewert 1991). Turtle and squamate species that experience embryonic diapause usually require a period of chilling before development can successfully recommence (Ewert & Wilson 1996; Booth 2002a; Andrews *et al.* 2008).

In contrast, delayed hatching and embryonic aestivation are strategies employed to prolong the residence of an embryo within the egg in response to *un*favourable environmental conditions (Ewert 1991; Ewert & Wilson 1996). During both forms of arrest, the embryo is in the final stages of development and typically remains within an unpipped egg (Thompson 1989; Ewert 1991). Delayed hatching occurs in crocodile, squamate and turtle groups and usually persist for several weeks in reptile eggs (Ewert 1991; Doody 2011). On the contrary, aestivation is a late-embryonic dormancy that may last for weeks or months and empirical evidence of its occurrence

has only been documented in turtles, although it may also occur in crocodiles and lizards (Auffenberg 1988; Thorbjarnarson & Hernandez 1993; Doody *et al.* 2001; Doody 2011). Both delayed hatching and aestivation are thought to occur in response to temperature and provide strategies to coincide hatching with the onset of early wet-season productivity to promote growth and survival (Webb *et al.* 1986; Thompson 1989; Ewert 1991). Similar to embryonic diapause, a trigger is needed to stimulate pipping and hatching and in its absence embryos become weak and eventually die if residual energy reserves become depleted (Ewert 1991; Horne 2007).

Finally, cold torpor is the suspension of development that embryos use to survive brief periods during temperatures that are too low to support developmental requirements (Ewert 1985; Hubert 1985; Ewert 1991). The embryos of most species of reptile are capable of using this type of arrest at any stage during incubation, although others such as the montane lizard, *Acritoscincus duperreyi*, can only enter cold torpor when they are full term embryos and not during earlier stages of development (Radder & Shine 2006). It is a direct response to unfavourable weather and in many cases it protects embryos for varying durations in below-critical temperatures, although the chances of mortality increase with increased duration spent in torpor (Christian *et al.* 1986; Ewert 1991). Cold torpor can maintain pre-ovipositional arrest in turtle embryos (Harry & Limpus 1989) and in other amniote species it can prevent development of embryos after oviposition until all eggs in the clutch have been laid (Welty 1982). This allows the synchronisation of development and hatching of the clutch in birds (Hébert 2002).

Within natural turtle nests, temperature varies spatially (Booth & Astill 2001; Wallace *et al.* 2004), resulting in asynchronous developmental rates and uneven patterns of hatching success (Houghton & Hays 2001; Wallace *et al.* 2004; Santidrian Tomillo *et al.* 2012). Therefore, the position of the egg within the nest influences the temperature that it experiences, possibly having differential effects on the breaking of pre-ovipositional arrest or the onset of subsequent types of arrest post-oviposition, within a clutch (Wallace *et al.* 2004; Ralph *et al.* 2005). Additionally, the types of embryonic arrest post-oviposition seem to vary among species in turtles, but pre-ovipositional arrest in this reptile group is universal and probably a process conserved during evolutionary time. This suggest that perhaps all turtle species also possess remnant traces of an ability to arrest development post-oviposition, or perhaps may be pre-adapted to evolve it.

The primary objectives of this comparative study were to test the hypotheses that egg incubation temperature influences embryonic developmental arrest, and when embryonic mortality occurs.

We achieved this by observing embryonic development at three different incubation temperatures in eggs of the green sea turtle, *Chelonia mydas*, and 3 species of freshwater turtle; the western oblong turtle, *Chelodina oblonga*, the eastern longneck turtle, *Chelodina longicollis*, and the Murray River turtle, *Emydura macquarii*. As mentioned earlier, all turtle embryos are laid early in the development schedule as arrested gastrulae (Ewert 1985; Miller 1985), allowing comparisons of development to be easily made among temperature treatments. We incubated eggs under conditions of low, medium, and high temperature from the time of oviposition until either hatching occurred or eggs were determined to be dead. First, we evaluated the response of embryos in a state of pre-ovipositional arrest to differences in temperature immediately after oviposition. We then assessed whether temperature influenced the developmental stage of embryonic mortality. We anticipated that mortality would be pronounced at specific developmental stages, presumably because at constant temperatures embryos remain arrested in the absence of a stimulus to recommence development or pip.

5.2 Methods

5.2.1 Study animals and egg collection

Gravid *C. oblonga* were trapped from Lake Goolelall in Western Australia, between October 1st and 7th, 2010, using baited, modified funnel traps, while gravid *C. longicollis* and *E. macquarii* were trapped from Lake Coranderrck, Victoria, between October 10th and December 15th, 2010, using baited fyke nets. Detection of gravid freshwater females was through manual palpation of the inguinal pocket. Those determined to be gravid using this method were later radiographed to confirm egg presence. All gravid freshwater females were induced to lay in the laboratory using an intramuscular injection of synthetic oxytocin (Butocin, Bumac Pty Ltd., NSW, Australia) at a dose of 15 IU / Kg (Ewert & Legler 1978), with oviposition generally occurring within 20 minutes. *C. mydas* eggs were collected from two females during oviposition on Heron Island, Australia, in December, 2010. All animals and resulting hatchlings were released alive at their point of origin at the conclusion of this investigation.

5.2.2 The breaking of pre-ovipositional arrest

Upon oviposition, all eggs were patted dry with a paper towel and weighed using an electronic balance (±0·1 g) to obtain egg wet mass. Egg maximum length and maximum width (±0.01 mm)

was measured using digital callipers and the egg was marked on the shell for identification using a graphite pencil. *C. mydas* eggs that were collected in the field were transferred immediately to a refrigerator and cooled for 10 hours at <10°C to halt embryonic development (Harry & Limpus 1989). They were then transported by sea, land and air in a 58 L sealed container filled with expanded polystyrene pellets, to the Monash University Animal Housing Facility, Melbourne. Transportation time was approximately 22 hours and temperature in the container did not exceed 14 °C, determined using a temperature data logger (model SL51T, Signatrol Ltd, England). Freshwater turtle eggs were processed immediately upon oviposition in the laboratory.

Eggs from all females of each species were randomly distributed among three temperatures specific to each species and devised from temperature ranges reported in the literature for natural nests (Table 1). Treatments were chosen to represent the nest temperatures possibly experienced by eggs at, and for several hours after oviposition. Thermal experimental design was based on incubation studies previously described (Booth 2000). All eggs were placed in separate plastic containers with loosely fitting lids containing air holes, with approximately half the shell covered in moist sand with a water potential of approximately -150 kPa (determined by thermocouple psychrometry), which was maintained weekly. Each treatment group was placed in a separate but identical GQF hovabator incubator (model 1632; Grandview Management Pty Ltd., WA, Australia) at their designated constant temperature (Table 5.1). For *Chelonia mydas* eggs, the treatment time was considered to begin when each group was placed in an incubator after transportation.

Due to the small number of eggs and the limited number of incubators available for the study, thermal treatments could not be replicated for each species. Although it is possible to confound treatment effects with incubator effects using this design (Packard & Packard 1993), it is unlikely that this was the case, because clutch and treatment usually have such large effects that they overwhelm any other effects in turtle egg incubation experiments (Packard & Packard 1993; Booth 2000). In addition, the use of identical incubation equipment was also likely to keep incubator effects to a minimum. Therefore the likelihood of confounding treatment effects with incubator and container effects was minor, so it was assumed that the only difference among incubators was the temperature.

Table 5.1. Incubation temperatures experienced by eggs of the green sea turtle (*Chelonia mydas*), the western oblong turtle (*Chelodina oblonga*), the eastern longneck turtle (*Chelodina longicollis*), and the Murray River turtle (*Emydura macquarii*) during each treatment.

Species	Temperature treatment		
Chelodina oblonga[1]	16°C	22°C	28°C
Chelodina longicollis[2]	16°C	22°C	28°C
Emydura macquarii[3]	16°C	22°C	28°C
Chelonia mydas[4]	24°C	28°C	32°C

[1]Clay (1981) (14°C – 29.8°C);

[2]Goode and Russell (1968) (14.4°C – 29.6°C)

[3]Goode and Russell (1968) (14.4°C – 29.6°C); Thompson (1988) (16.9°C - 27°C)

[4]Booth and Astill (2001) (24°C – 34°C)

Eggs were checked twice daily after the commencement of each treatment for the formation of the characteristic white spot, considered to be the first visual sign that pre-ovipositional arrest has broken and the egg is developing (Thompson 1985; Booth 2002b). Observations continued until all eggs had either developed a white spot or were determined to be dead (discoloured or smelling). All *C. oblonga* eggs in treatment one (16ºC) failed to develop white spots so were transferred to 28ºC after 38 days. Two eggs subsequently recommenced development, one of which hatched, after incubating at 28ºC for the duration of development (126 days lapsed from white spot development to hatching). All eggs in this treatment were excluded from analysis.

5.2.3 Embryonic development and hatching

Eggs were checked daily from the time of white spot development until hatching and the duration of incubation (days lapsed from white spot development to hatching) calculated. Once hatched, adhering sand was brushed free from each hatchling and they were placed in a plastic container with a moist towel and allowed to absorb their residual yolk for 24-48 hours. Hatchlings were then weighed using an electronic balance (±0·1 g) to obtain hatchling mass, and measurements of the carapace length and width, plastron length and width, and head width were also taken using digital callipers (±0.01 mm).

5.2.4 Embryonic mortality

For all species, eggs that failed to develop a white spot or were determined to be dead during incubation were dissected and the developmental stage at death was determined using the staging

criteria previously described for freshwater (Yntema 1968) and marine (Miller 1985) turtle embryos.

5.2.5 Data analysis

The proportion of eggs at each temperature to form a white spot was calculated from the total number of eggs in the temperature treatment and then compared for each species using Pearson's Chi-squared test. An ANCOVA was used to identify whether temperature affected the time elapsed (days) between treatment commencement and white spot formation. *C. oblonga* eggs at 16ºC were excluded from both aforementioned statistical tests because all eggs failed to form a white spot at this temperature. Pearson's Chi-squared test was also used to identify if there was a significant difference in hatching success between 28ºC and 32ºC for *C. mydas*. Non parametric Mann–Whitney *U*-tests were then applied to *C. mydas* hatchling parameters for both aforementioned temperature treatments. Low sample size prevented these analyses for freshwater turtles. Data analysis was conducted in R, statistical package 2.11.0 (R Development Core Team 2010) and two tailed $P \leq 0.05$ was considered statistically significant. Data presented are mean ± standard error.

5.3 Results

5.3.1 The breaking of pre-ovipositional arrest

In total, 97 eggs from two *C. mydas*, 59 eggs from seven *C. oblonga*, 105 eggs from six *E. macquarii* and 25 eggs from four *C. longicollis* females were used in this study. The distribution of eggs among temperatures for each species is shown in Table 5.2.

Successful recommencement of development and the formation of white spots were seen at each temperature, except at 16ºC for *C. oblonga*, in which all eggs failed to form a white spot. Although, there was no significant difference in the proportion of eggs to form white spots at each temperature for *C. mydas* ($X^2 = 0.01$, $P = 0.99$), *C. oblonga* ($X^2 = 0.30$, $P = 0.58$), *E. macquarii* ($X^2 = 3.60$, $P = 0.17$) or *C. longicollis* ($X^2 = 0.05$, $P = 0.98$), a variation among treatments was observed (Table 5.2). For example, the proportion of eggs to form white spots was lowest for *C. mydas*, *C. oblonga* and *E. macquarii* at the lowest temperature (96.9% at 24ºC, 0% at 16ºC and 46.88% at 16ºC respectively), while the converse was true for *C. longicollis*, with 100% of eggs forming white

spots at 16ºC (Table 5.2). Additionally, the proportion of eggs to form white spots was equal at 28ºC and 32ºC for *C. mydas* (100%) and at 22ºC and 28ºC for *C. longicollis* (87.50%), whereas the success of *C. oblonga* and *E. macquarii* eggs tended to decrease with the increase in temperature between these two latter temperatures (Table 5.2).

Table 5.2. Effects of temperature treatment on days to white spot formation (representing the time to break pre-ovipositional arrest) and the proportion of eggs to successfully recommence development (represented by the development of the white spot).

Species	**Temperature treatment**		
	24°C	28°C	32°C
Chelonia mydas			
Sample size	32	32	33
Proportion of eggs to form white spot	96.9% (31)	100% (32)	100% (33)
Time to formation of white spot	1.29 ± 0.08	1.25 ± 0.08	1.27 ± 0.19
	16°C	22°C	28°C
Chelodina oblonga			
Sample size	20	19	20
Proportion of eggs to form white spot	0.00% (0)	78.95% (15)	63.16% (12)
Time to formation of white spot	-	11.47 ± 1.72	12.83 ± 3.60
Emydura macquarii			
Sample size	32	42	31
Proportion of eggs to form white spot	46.88% (15)	95.24% (40)	83.87% (26)
Time to formation of white spot	4.53 ± 0.90	3.10 ± 0.50	3.30 ± 0.70
Chelodina longicollis			
Sample size	9	8	8
Proportion of eggs to form white spot	100.00% (9)	87.50% (7)	87.50% (7)
Time to formation of white spot	3.78 ± 0.70	2.57 ± 0.78	1.71 ± 0.29

Likewise, temperature had no effect on the time to white spot formation for *C. mydas* ($F = 0.02$, $P = 0.98$), *C. oblonga* ($F = 0.13$, $P = 0.72$), *E. macquarii* ($F = 1.06$, $P = 0.353$) or *C. longicollis* ($F = 2.66$, $P = 0.09$) (Table 5.2), with only marginal differences being observed among temperatures for each species. Generally, *C. oblonga* eggs took almost three times as long to recommence development and form white spots at 22ºC and 28ºC, than eggs of the other species, with periods of 11.47 ± 1.72 and 12.83 ± 3.60 days being recorded, respectively (Table 5.2). On the other hand, *C. mydas* eggs developed white spots at each temperature sooner than eggs of all other species with periods of 1.29 ± 0.08, 1.25 ± 0.08 and 1.27 ± 0.19 days at 24ºC, 28ºC and 32ºC respectively (Table 5.2).

5.3.2 Embryonic development and hatching

Incubation time decreased for each species as the temperature increased (Table 5.3). *C. mydas* eggs hatched soonest at 32ºC (55.69 ± 0.09 days), followed by 28ºC (72.25 ± 0.41 days) and then 24ºC (121.50 ± 0.50 days) (Table 5.3). Similarly, incubation time of *E. macquarii* and *C. longicollis* eggs at 22ºC (116.17 ± 0.40 and 111.25 ± 0.75 days respectively) was almost double the time observed at 28ºC (51.50 ± 0.98 and 61.17 ± 0.60 days respectively). This was also the case for *C. oblonga* eggs, which generally had longer incubation times at each temperature than the other three species (Table 5.3).

With the exception of two *C. mydas* eggs, all eggs of all species failed to hatch at the lowest temperature. Further, the hatching success was generally quite poor for *C. oblonga*, *E. macquarii* and *C. longicollis* eggs at 22ºC (10.53%, 14.29% and 50.00% respectively) but increased at 28ºC (20%, 45.16% and 75% respectively) (Table 5.4). *C. mydas* showed the highest hatching success of all species at both 28ºC (87.50%) and 32ºC (87.90%), although there was no significant difference between these temperatures ($X^2 < 0.01$, $P = 0.99$).

C. mydas hatchlings had similar plastron ($W = 363.50$, $P = 0.50$) and head widths ($W = 481.00$, $P = 0.23$) at 28ºC and 32ºC (Table 5.3). However, hatchling mass ($W = 608.50$, $P < 0.01$), carapace length ($W = 646.50$, $P < 0.01$), carapace width ($W = 804.50$, $P < 0.01$) and plastron length ($W = 583.00$, $P < 0.01$) differed significantly between these temperatures. Therefore, when incubated at a lower temperature at 28ºC, hatchlings were significantly heavier, had longer and wider carapaces, and longer plastrons than those that were incubated at 32ºC (Table 5.3). Although statistical significance could not be established for the differences observed in freshwater hatchling parameters among temperatures, the higher temperature at 32ºC generally resulted in larger hatchlings than 28ºC, with the exception of hatchling plastron width for *C. oblonga*, head width for *E. macquarii* and both plastron length and head width for *C. longicollis* (Table 5.3).

Table 5.3. Effects of temperature treatment on incubation duration (mean days ± standard error) from white spot formation to hatching, and hatchling morphology.

Species	Temperature treatment		
	24°C	28°C	32°C
Chelonia mydas			
Sample size	2	28	29
Incubation time	121.50 ± 0.50	72.25 ± 0.41	55.69 ± 0.09
Hatchling mass (g)	25.93 ± 0.83	29.10 ± 0.34	27.62 ± 0.25
Carapace length (mm)	49.00 ± 1.00	50.96 ± 0.24	49.68 ± 0.21
Carapace width (mm)	39.50 ± 0.50	39.39 ± 0.21	36.82 ± 0.15
Plastron length (mm)	35.50 ± 1.50	40.38 ± 0.22	39.29 ± 0.25
Plastron width (mm)	35.50 ± 1.50	24.80 ± 0.20	24.98 ± 0.35
Head width (mm)	15.60 ± 0.10	15.92 ± 0.08	15.76 ± 0.07
	16°C	22°C	28°C
Chelodina oblonga			
Sample size	0	2	4
Incubation time	-	151.00 ± 10.00	117.50 ± 5.33
Hatchling mass (g)	-	4.72 ± 0.88	5.28 ± 0.22
Carapace length (mm)	-	24.80 ± 1.90	28.43 ± 0.43
Carapace width (mm)	-	18.35 ± 2.75	20.95 ± 0.38
Plastron length (mm)	-	20.75 ± 0.45	22.53 ± 0.79
Plastron width (mm)	-	13.60 ± 1.70	12.63 ± 0.55
Head width (mm)	-	9.90 ± 0.40	10.35 ± 0.15
Emydura macquarii			
Sample size	0	6	14
Incubation time	-	116.17 ± 0.40	51.50 ± 0.98
Hatchling mass (g)	-	4.89 ± 0.26	4.98 ± 0.30
Carapace length (mm)	-	28.15 ± 0.53	29.24 ± 0.71
Carapace width (mm)	-	24.55 ± 1.06	26.66 ± 0.79
Plastron length (mm)	-	23.10 ± 0.49	24.05 ± 0.58
Plastron width (mm)	-	12.48 ± 0.72	13.21 ± 0.37
Head width (mm)	-	9.53 ± 0.30	9.21 ± 0.21
Chelodina longicollis			
Sample size	0	4	6
Incubation time	-	111.25 ± 0.75	61.17 ± 0.60
Hatchling mass (g)	-	3.72 ± 0.38	4.21 ± 0.16
Carapace length (mm)	-	24.70 ± 0.68	25.02 ± 1.76
Carapace width (mm)	-	19.78 ± 0.81	19.93 ± 1.78
Plastron length (mm)	-	19.65 ± 0.90	19.25 ± 1.67
Plastron width (mm)	-	14.25 ± 0.50	14.97 ± 1.75
Head width (mm)	-	8.30 ± 0.06	7.77 ± 0.78

Table 5.4. Effects of temperature treatment on hatching success.

Species	Temperature treatment		
	24°C	28°C	32°C
Chelonia mydas			
Sample size	32	32	33
Number of eggs to hatch	2	28	29
Hatching success	6.30%	87.50%	87.90%
	16°C	22°C	28°C
Chelodina oblonga			
Sample size	20	19	20
Number of eggs to hatch	0	2	4
Hatching success	0.00%	10.53%	20.00%
Emydura macquarii			
Sample size	32	42	31
Number of eggs to hatch	0	6	14
Hatching success	0.00%	14.29%	45.16%
Chelodina longicollis			
Sample size	9	8	8
Number of eggs to hatch	0	4	6
Hatching success	0.00%	50.00%	75.00%

5.3.3 Embryonic mortality

Figs 1 – 4 show the mean percentage of embryos that died at each developmental stage at each temperature for each species. Percentage mortality was calculated at each stage for each species as a proportion of the total number of eggs that died at each temperature. *C. mydas* eggs predominantly died at 24ºC during late stages of development with a peak of 14 embryos dying at stage 30, representing 45% of the observed mortality at that temperature (Fig. 1). Both *C. oblonga* and *E. macquarii* had similar bimodal mortality patterns, with peaks in embryonic death occurring at 16ºC at stage 0 (95% (18 eggs) and 53% (17 eggs) of mortality respectively), and at 22ºC at stage 25 (29% (5) and 64% (23) of mortality respectively) (Figs 2 and 3). At 28ºC, peaks in embryonic mortality of *C. oblonga* and *E. macquarii* embryos occurred at both stage 0 (60% (9) and 24% (5) of mortality respectively) and 25 (27% (4) and 43% (9) mortality respectively) (Figs 2 and 3). There was no apparent pattern in embryonic mortality in *C. longicollis* (Fig. 4).

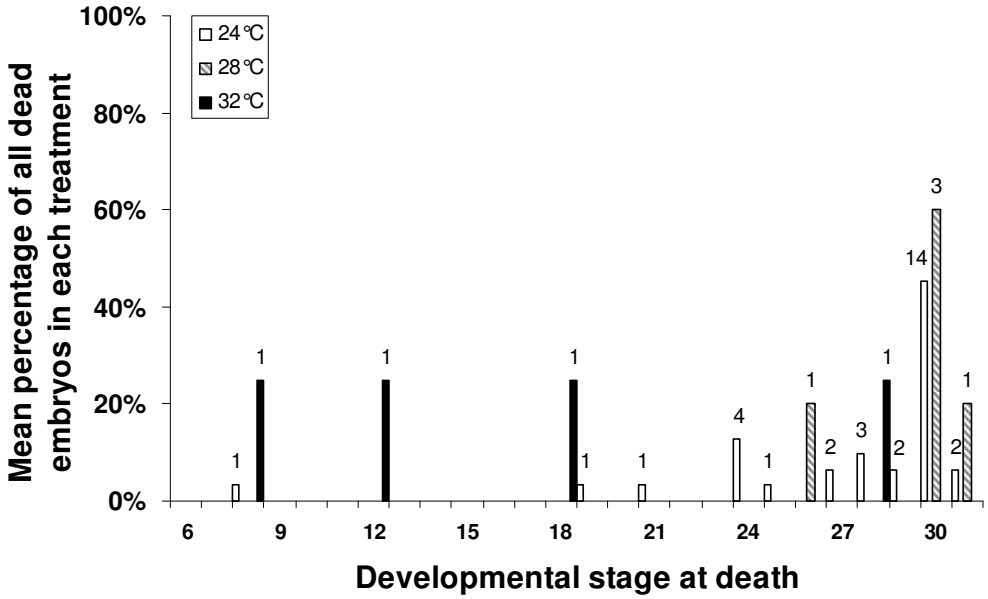

Figure 5.1. The mean percentage of *Chelodina mydas* embryos that died at each developmental stage in the 24°C treatment (n=31; 29 pre-hatching and 2 post-hatching), 28°C treatment (n=5; 4 pre-hatching and 1 post-hatching) and 32°C treatment (n=4). Values reported above each bar represent the absolute number of embryos that died at that stage in the treatment.

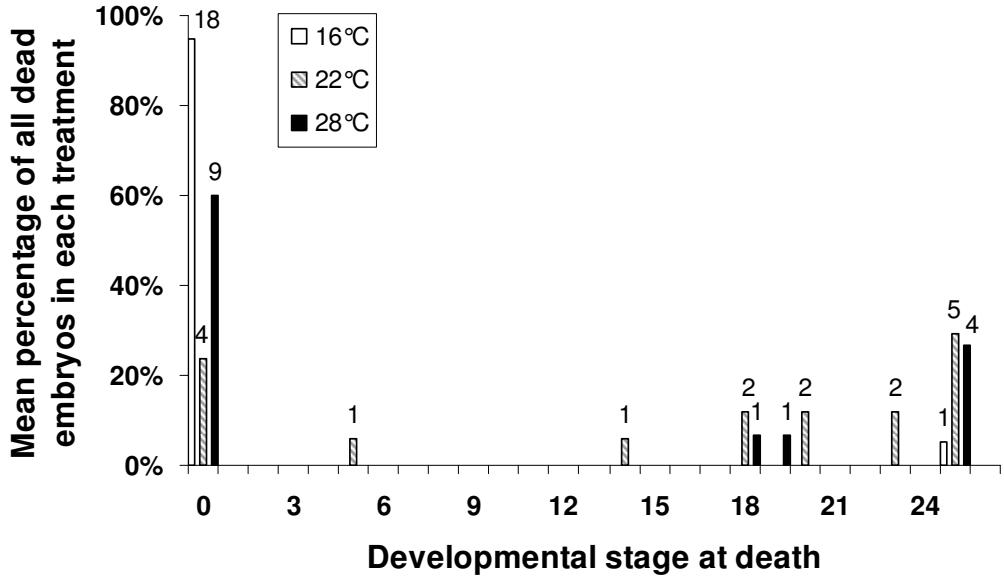

Figure 5.2. The mean percentage of *Chelodina oblonga* embryos that died at each developmental stage in the 16°C treatment (n=19), 22°C treatment (n=17) and 28°C treatment (n=15). Values reported above each bar represent the absolute number of embryos that died at that stage in the treatment.

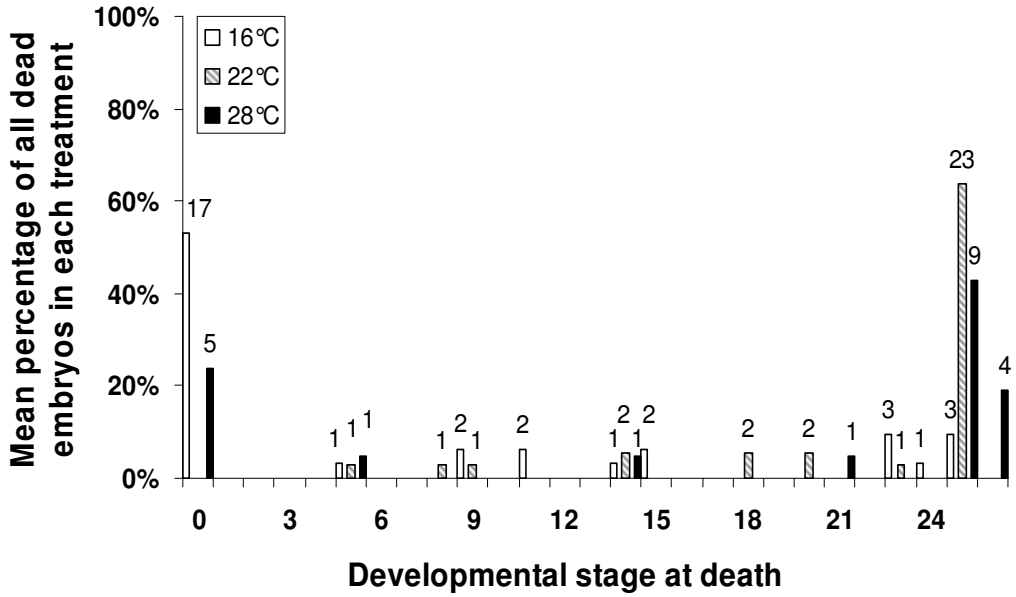

Figure 5.3. The mean percentage of *Emydura macquarii* embryos at each developmental stage in the16°C treatment (n=32), 22°C treatment (n=36) and 28°C treatment (n=21; 17 pre-hatching and 4 post-hatching). Values reported above each bar represent the absolute number of embryos that died at that stage in the treatment.

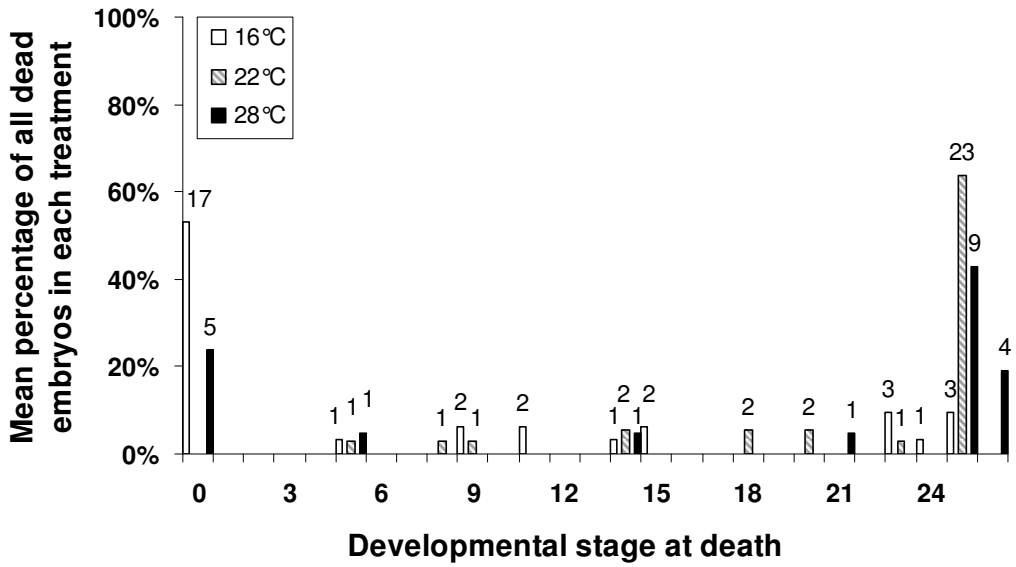

Figure 5.4. The mean percentage of dead *Chelodina longicollis* embryos that died at each developmental stage in the 16°C treatment (n=9), 22°C treatment (n=4) and 28°C treatment (n=2). Values reported above each bar represent the absolute number of embryos that died at that stage in the treatment.

5.4 Discussion

Our study shows that neither the proportion of eggs to form a white spot nor the time to white spot development varied with temperature for the four turtle species under investigation. This suggests that the breaking of pre-ovipositional arrest after oviposition occurred independently of temperature, which is surprising considering that reptilian development is positively correlated with temperature during earlier rather than later stages (Webb 1987; Andrews 2004). In the lowest temperature treatment, the breaking of pre-ovipositional arrest and the recommencement of development occurred despite an incubation temperature that was lower than needed to facilitate successful embryonic development through to hatching. This was also observed in *C. expansa* eggs, although eggs of this species subsequently undergo a period of embryonic diapause, arresting embryos to presumably protect them during sub-optimum incubation temperatures (Booth 2000). None of the eggs in our study appeared to experience embryonic diapause, because (1) once white spot formation occurred, full chalking of the eggshell followed and vitelline circulation was evident, and (2) the absence of a thermal cue to break diapause at constant temperatures would likely have resulted in an increase in embryonic mortality between gastrulation and somite development in diapaused embryos, and this was not observed (Ewert 1985). Therefore, if the breaking of pre-ovipositional arrest occurs at low temperatures when embryos are incapable of subsequently entering embryonic diapause, survival at early stages may be compromised if low temperatures persist.

Our results agree with previous findings that show white spot formation generally occurs between 1-4 days post-oviposition in turtles (Miller 1982; Ewert 1985; Miller 1985; Thompson 1985; Chan 1989). However, the time to white spot formation in *C. oblonga* was noticeably longer than the other three species under investigation and relates more closely to developmental patterns observed in *C. expansa* (Booth 2000; Booth 2002a). Cold torpor may have maintained pre-ovipositional arrest at 16ºC (Harry & Limpus 1989) although it is unlikely that this was the case at 22ºC and 28ºC. Furthermore, a significant proportion of embryonic mortality occurred prior to white spot formation at all three temperatures for *C. oblonga*, suggesting that embryos are failing to break pre-ovipositional arrest at constant temperatures, perhaps because they require an environmental cue after oviposition in order to recommence development.

Several studies suggest that the environment that embryos experience *in utero* influences the persistence of pre-ovipositional arrest post-oviposition in addition to embryo survival. Booth

(2002) proposed that prolonged periods of egg retention during which embryos remain arrested in the oviduct may result in shorter periods of arrest and time to white spot formation after eggs have been laid. Although this theory seems quite plausible based on observations of individual freshwater species that have protracted periods of pre-ovipositional arrest after oviposition, an assessment of the comparative literature implies that this is unlikely. For example, white spot formation in marine species is quite consistent and generally only takes up to 4 days despite egg retention periods that can range from 7-13 days in leatherback turtles (Rafferty *et al.* 2011) and up to 63 days in olive ridley turtles (Plotkin *et al.* 1997).

Additionally, Rafferty et al (2011) found that the duration of pre-ovipositional arrest influences the stage that leatherback turtle embryos die as well as overall clutch hatching success. Prolonged periods of pre-ovipositional arrest also results in developmental deformity and death of freshwater turtle embryos (Ewert 1985). Presumably, turtle embryos become arrested *in utero* in response to an unknown factor that appears to have detrimental effects on the embryo during periods of prolonged exposure. Although further research is needed to identify what this limiting factor may be, it is likely that oviducal hypoxia is the cause (Rafferty & Reina 2012).

Furthermore, maternal body temperature during key reproductive stages is thought to influence the expression of embryonic diapause. The striped mud turtle, *Kinosternon baurii*, that nest twice yearly during both spring and autumn only produce embryos that express embryonic diapause during autumn (Ewert & Wilson 1996). The 'decision' of whether or not an embryo expresses embryonic diapause is thought to be determined by the temperature that mothers experience during late oogenesis (between ovulation and fertilization) and if females of this species undergo chilling during this sensitive period, their embryos do not express embryonic diapause post-oviposition, but the embryos of unchilled females do (Ewert & Wilson 1996). Determining maternal effects, defined as the underlying pressure exerted by the maternal genotype or phenotype that influences the offspring phenotype (Wolf & Wade 2009), may hold the key to fully understanding how and why arrested development occurs.

It is well known that temperature can have profound physiological effects on embryonic development in reptiles (Deeming & Ferguson 1991) and during our investigation temperature influenced incubation duration, egg mortality and hatchling phenotype. Firstly, our findings support existing evidence that an increase in incubation temperature, within desirable ranges, generally increases embryonic metabolic activity and reduces incubation period (Deeming &

Ferguson 1991). Incubation times were consistent with those reported during artificial incubation studies for *C. mydas* (Booth & Astill 2001), *E. macquarii* and *C. longicollis* (Goode & Russell 1968). However, laboratory incubation studies have not been conducted to look at embryonic development in *C. oblonga* eggs, but published observations on the reproductive habits of this species reported natural incubation periods between 210 – 222 days (Clay 1981). The minimum incubation period (from white spot to hatching) in this study was approximately 115 days and in addition to the absence of embryonic diapause during incubation, it is plausible to conclude that eggs are possibly hatching prior to winter and that hatchlings are overwintering in the nest before emergence. This process has been observed in several other species of freshwater turtle (Costanzo *et al.* 2004; Ultsch & Reese 2008).

To our knowledge, this is the first comparative study to report the direct effects of temperature on the specific developmental stage at which turtle embryos die. During our investigation, embryonic mortality appeared to occur predominantly at either early (eggs failed to resume development after oviposition) or late (just prior to hatching) stages of development. Interestingly, the majority of *E. macquarii* embryos at 22ºC completed embryonic development but failed to pip the egg and hatch. Similarly, this was also the case for a large proportion of *C. mydas* embryos incubated at 24ºC that failed to pip and hatch after completing development. Presumably, this may have occurred because embryos of each species entered a state of delayed hatching, awaiting a necessary stimulus to trigger pipping that never came (Thompson 1989; Ewert 1991). This has been proposed previously for *E. macquarii* (Thompson 1989; Ewert 1991), but this is the first evidence in *C. mydas* and indicates that sea turtles possibly retain remnant traces of an ability they once possessed, or perhaps that they may be pre-adapted to evolve delayed hatching in the future (Ewert 1991). Nevertheless, a considerable amount of further research is needed to identify whether this is the case.

In natural *E. macquarii* nests, daily variations in temperature of up to 12°C are typically observed (Thompson 1988) and in several species of turtle these fluctuations in temperature are the necessary cue to induce pipping, hatching and nest emergence (Ewert 1991; Gyuris 1993). The full term *E. macquarii* embryos incubated at 22ºC were at a constant temperature higher than the daily average observed in natural nests (Thompson 1988) so presumably a decrease in temperature was needed to stimulate pipping. Conversely, *C. mydas* embryos incubated at 24ºC are likely to have needed an increase in temperature to stimulate pipping, although this is speculative and needs further investigation. Even so, our findings do suggest that *C. mydas*

embryos may be pre-adapted to possess delayed hatching in instances of undesirable nest temperature, possibly choosing to remain in the egg during these periods because it provides a safer environment than if they were to hatch and remain in the nest cavity.

Further, large thermal gradients in reptile nests are known to create independent developmental conditions for each egg, thus creating intra-clutch variation in incubation period (Andrews 2004). However, synchronous hatching still occurs in many species through alteration of developmental rate, allowing underdeveloped eggs to "catch up" with more developed siblings and hatch together (Thompson 1989). This has been demonstrated in *E. macquarii* embryos, capable of changing their metabolic and heart rates to hasten development and hatch with more advanced siblings (McGlashan *et al.* 2011). Clearly, species such as *E. macquarii* possess a diverse array of strategies to alter their developmental rates so that pipping and hatching occurs when conditions are most conducive to survival. It is therefore plausible that a continuum exists during which embryos are capable of either hatching early or late in order to optimise their chance of success.

Finally, significant differences in *C. mydas* hatchling parameters were observed between 28ºC and 32ºC as a result of incubation temperature. Incubation temperature has the ability to affect hatchling phenotype in reptiles (Booth 2006) and possibly impact on the ultimate fitness of an animal (Andrews 2008). It would therefore be interesting to identify if temperature induced periods of arrested development impact on hatchling fitness and success by employing a long-term study approach.

In conclusion, it is evident there is a large inter-specific variation in strategies used by reptile embryos that enable them to withstand undesirable conditions during development. We are only beginning to appreciate how resilient reptile embryos are and more research is needed to understand their capacity to time development and hatching to coincide with environmental conditions that promote optimum growth and survival, despite the absence of parental care.

5.5 Acknowledgements

Thanks to Danielle Stockheld, Peter Comber, Gerald Kuchling, Jacqueline Giles, Bob Prince, Roger Evans and Belinda Lees for their valuable help and advice. This study was made possible with support from Franciscus Scheelings, Bryan Tormey, Jason Van Rijn, Zoos Victoria, and the staff of the Healesville Sanctuary Wildlife Health Centre and the Heron Island Research Station.

Research was conducted under scientific permits issued by the Queensland Environmental Protection Agency (WITK0906210 and WISP08063210), the Victorian Department of Sustainability and the Environment (10005293), and the Western Australian Department of Environment and Conservation (SF007435; CE002893) and was approved by the Animal Ethics Committee of Monash University (BSCI/2009/23 and BSCI/2009/28). This work was supported by the Holsworth Wildlife Foundation and academic funding provided by Monash University.

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Oblonga turtle (*Chelodina oblonga*) eggs showing various stages of eggshell "chalking"
Photo taken by Anthony R. Rafferty

Top: Hovabator incubators used during the temperature study
Bottom: A hatchling green turtle (*Chelonia mydas*) in its holding tank
Photos taken by Anthony R. Rafferty (Top) and Rohan Clarke (Bottom)

Chapter Six

Maternal health influences reproductive investment in three species of freshwater turtle

Anthony R. Rafferty[1], T. Franciscus Scheelings[2] & Richard D. Reina[1]

[1]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

[2]Australian Wildlife Health Centre, Healesville Sanctuary, Healesville, Victoria, Australia

In review under the same name in Functional Ecology

Submitted 8th May 2012

Declaration for Thesis Chapter Six

Monash University

Declaration by candidate

In the case of Chapter six, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conception, initiation, design, ethics, permits, data collection and analysis, project coordination and manuscript preparation	90%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
T. Franciscus Scheelings	Data collection and manuscript editing	5%
Richard Reina	Supervision, manuscript editing and direction	5%

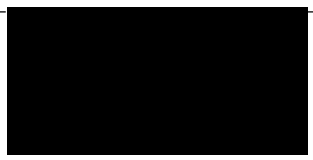
Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Monash University, School of Biological Sciences, Clayton campus

	Signature	**Date**
T. Franciscus Scheelings		09/05/2012
Richard Reina		

Oblong turtle (*Chelodina oblonga*) eggs, including one yolkless egg, after oviposition
Photo taken by Anthony R. Rafferty

Abstract

1. Life-history theory predicts that a trade-off in the allocation of resources among different physiological systems exists because resources are finite. As a result, females in poor health may choose to invest in self-maintenance rather than reproduction.
2. We use haematology, serum biochemistry and morphometric measurements as indicators of physiological health state to investigate whether maternal health influences reproductive investment in three Australian freshwater turtles; the oblong turtle (*Chelodina oblonga*, $n = 12$), the Macqaurie turtle (*Emydura macquarii*, $n = 9$) and the eastern long-necked turtle (*Chelodina longicollis*, $n = 8$).
3. Females of all three species altered their level of reproductive investment based on health state, manifested by changes in egg mass and size, as well as clutch size, although different trade-offs were observed among species. These findings agree with the physiological constraint hypothesis. Furthermore, no trade-offs existed between clutch and egg size in any of the species under investigation, refuting the optimum egg size hypothesis and agreeing with previous findings in other chelonian species.
4. Haematological and biochemical indices of health state differed among species. Haematological measures of hematocrit, total leucocyte count, and heterophil/lymphocyte ratio only related to reproductive investment in *E. macquarii*, whereas analyses of total plasma protein, glucose, albumin/globulin ratio and aspartate transaminase generally correlated with numerous reproductive measures in all species.
5. Carapace length was a better predictor of reproductive investment than body condition, although further investigation is needed to identify whether morphometric measurements are appropriate tools in assessing physiological state.
6. This study highlights the connection between life-history evolution and animal physiology by documenting for the first time, how measures of physiological health state relate to reproductive investment in Australian freshwater turtles. Our results emphasize the need to investigate the role that maternal health plays in dictating the reproductive success of a species. Focusing on improving the health of mothers in addition to increasing hatching success in waning populations may prove successful for the conservation and management of threatened and endangered species.

6.1 Introduction

Reproduction is an energetically expensive process that involves the reallocation of resources that could otherwise be used by competing physiological systems (Harshman & Zera 2007). A trade-off between reproduction and the maintenance of good health may arise as a result and has been documented in several vertebrate species (Uller *et al.* 2006; Wagner *et al.* 2008; Norte *et al.* 2010). Accurate estimations of the physiological condition or health status of an animal can be made by assessing the haematology and plasma or serum biochemistry of the individual and such evaluations have proven useful tools in ecological studies (Artacho *et al.* 2007b; Artacho *et al.* 2007a).

Measures of haematology and biochemistry, along with body morphometrics, are readily applied in avian research and have been used to demonstrate that maternal health state influences reproductive investment and breeding success in the Nazca booby (*Sula granti*) (Apanius *et al.* 2008), burrowing parrots (*Cyanoliseus patagonus*) (Masello & Quillfeldt 2004), great tits (*Parus major*) (Ots *et al.* 1998; Norte *et al.* 2010), and Magellic penguins (*Sphenicus magellicus*) (Moreno *et al.* 2002). In contrast, haematology and biochemistry reference ranges for reptiles are scarce in the literature (Dessauer 1970; Stein 1996; Cambell 2006) and the influence of maternal health state on reproductive investment remains relatively unknown (Perrault *et al.* 2012). However, understanding this is important because maternal reproductive investment by oviparous reptiles is generally restricted to pre-ovipositional allocations to egg size and number because females do not usually provide parental care after they lay their eggs (Wallace *et al.* 2007). Therefore, offspring investment in these species is more closely related to the provision of material and energy towards egg production, contrary to the post-ovipositional investment provided by avian species (Cockburn 2006). Reptiles are therefore particularly good candidates to test the optimal egg size theory that predicts a trade-off between egg size and number (Smith & Fretwell 1974) as a result of the physiological constraint associated with finite resource allocation (Bowden *et al.* 2004).

We used three species of Australian freshwater turtle to (1) identify the most appropriate physiological variables to assess maternal health, (2) identify how physiological state influences reproductive investment, and (3) test the optimal egg size hypothesis by examining the relationship between egg size and clutch size in all three species. Specifically, we investigated the

relationship between maternal health and measures of reproductive investment (clutch size and mean egg mass, length and weight) by assessing the physiological variables described below.

6.1.1 Haematology

Reptilian blood consists of red (erythrocytes) and white (leucocytes) blood cells and haematology is the study of detecting conditions that affect these blood cells (Cambell 2006). During this study we assessed several measures of red and white blood cells as indicators of female health. Total erythrocyte count and hematocrit (PCV - the relative abundance of red blood cells in total blood volume) reflect the oxygen carrying capacity of the blood. A reduction in both variables is called anaemia (Dein 1986; Campbell 1995). Additionally, total leucocyte count (WBC) identifies the immune system status and presence of infection or inflammation (Campbell 1995). The primary function of leucocytes is to protect against various pathogenic antigens and an increase in leucocyte concentration is often seen during an immune response (Dein 1986). Heterophils and lymphocytes are two abundant white blood cell types and the heterophil / lymphocyte ratio (H/L ratio) is readily used as an indicator of stress, generally in response to physiological perturbation, limited food availability and infectious disease (Gross & Siegel 1983; Maxwell 1993). An increase in this ratio is known to occur in individuals exposed to these stressors (Ots *et al.* 1998). Finally, parasitaemia in female reptiles has also been correlated with reduced reproductive output (Schall 1982; Madsen *et al.* 2005).

6.1.2 Plasma or serum biochemistry

The physiological state of an animal is also reflected in the metabolites present in the blood (Artacho *et al.* 2007a). Metabolites including total protein (TP) and glucose (GLU) are used to asses the nutritional state of an individual and generally decrease during periods of starvation or fasting (Ots *et al.* 1998; Artacho *et al.* 2007a). Furthermore, albumin and globulin are key proteins involved in immune system function and assessment of the albumin / globlin ratio (A/G) ratio is a useful tool for determining the health status of an animal. Typically, unhealthy individuals will exhibit a lower A/G ratio than healthy individuals (Kawai 1973). Important protein-binding minerals, calcium and phosphorus, are also essential for normal bodily function and the relative abundance of calcium to phosphorus (calcium / phosphorous (Ca/P) ratio) can have significant effects on egg production and offspring survival in the greater sage grouse, *Centrocercus urophasianus* (Dunbar *et al.* 2005) and hatching success in the leatherback turtle, *Dermochelys coriacea*

(Perrault *et al.* 2012). Although Ca/P may vary among species, possibly in relation to diet, a balance in both calcium and phosphorous levels is needed (Dunbar *et al.* 2005; Cambell 2006). To complement plasma or serum protein investigations an evaluation of the concentration of plasma or serum enzymes including aspartate transaminase (AST) and creatine kinase (CK) are used to predict, and help differentiate, between liver and muscle damage respectively (Harr 2002). Elevated concentrations of both enzymes are characteristically observed in unhealthy or traumatized individuals.

6.1.3 Morphology

Body mass (BM) and structural size (measured as carapace length (CL)) are morphometric measurements easily obtained in turtles and a large variation in both variables can be observed at the intra-specific level, resulting from an individual's phenotypic plasticity in response to their surrounding environment (Rowe 1997). The large variation in individual body mass makes this variable difficult to interpret and for this reason many studies prefer to look at body condition (BC). BC is calculated from the linear regression of BM against CL and by expressing BC as a residual, it separates BM from CL. BC and CL have previously been linked to reproductive investment in turtles (Wilkinson *et al.* 2005; Rasmussen & Litzgus 2010).

6.2 Methods

Data were collected from three 3 species of freshwater turtle; the western oblong turtle, *Chelodina oblonga* (n = 12), the Macquarie turtle, *Emydura macquarii* (n = 9) and the eastern longneck turtle, *Chelodina longicollis* (n = 8). *C. oblonga* were trapped between October 1st and 7th, 2010, from Lake Goolelall in Western Australia, using baited, modified funnel traps, while *C. longicollis* and *E. macquarii* were trapped between October 10th and December 15th, 2010, from Lake Coranderrck, Victoria, using baited fyke nets. Upon collection, the inguinal fosa of each female was manually palpated to determine if gravid. Females thought to be gravid were later radiographed to confirm presence of eggs.

Once at the laboratory, females were manually restrained and 1 mL of blood was collected from the jugular vein of each animal using a 22-gauge needle attached to a 3 ml syringe. Blood was immediately transferred to a plain tube (BD Microtainer™ Tubes, Vacutainer Systems, Franklin Lakes, New Jersey 07417, USA) for centrifugation and the resulting serum analysed using the

avian-reptilian rotar on the Vet Scan analyser (Abaxis Inc. Union City, California 94587, USA). PCV was determined using standard centrifugation in microhaematocrit tubes (Iris Sample Processing, Westwood, Massachusetts, 02090, USA).

Blood smears were air-dried and stained with Romanowsky stain (Rapid Diff, Australian Biostain Pty Ltd, Traralgon, Victoria 3844, Australia) before being examined to identify and assess the prevalence of hemoparasites within erythrocytes. This was determined by counting 1000 erythrocytes under 1000 X magnification with oil immersion and identifying how many were infected. We considered the intensity of blood parasite infection as an index of parasitemia for each individual.

WBC counts were performed manually using a haemocytometer. Differential leucocyte counts were obtained by examining 100 leucocytes under oil immersion. Heterophils and lymphocytes were present in the largest quantities and are the only immune cells included in this study. The relative abundance of both heterophils and lymphocytes were analyzed using the H/L ratio.

After blood collection, female *C. longicollis* were placed in individual 68 L (60 cm x 38 cm x 40 cm) containers and female *C. oblonga* and *E. macquarii* in individual 150 L (92 cm x 41.5 cm x 32.5 cm) containers so that each female was floating in enough water (28°C) to cover the shell. After allowing 1 hour for the turtles to acclimatise to their surroundings, they were given an intramuscular injection of synthetic oxytocin (Butocin, Bumac Pty Ltd., NSW, Australia) at a dose of 15 IU / Kg (Ewert & Legler 1978). Successful induction of oviposition generally occurred within 20 minutes.

As eggs were laid, they were immediately patted dry with a paper towel and weighed using an electronic balance (± 0·1 g) to obtain egg-wet mass (EM). Maximum egg length (EL) and width (EW) was also measured using a digital calliper (± 0·01 mm) based on standard procedure (Rasmussen & Litzgus 2010). Clutch size (CS; the total number of eggs laid in each clutch) was also noted for each female. Additionally, non-gravid female body mass (BM) was obtained post-oviposition using an electronic balance (kg; ± 0·1 g) and the midline carapace length (CL) was measured and recorded (± 0·01 cm).

All statistical analyses were conducted using R, statistical package 2.11.0 (R Development Core Team 2010). Egg morphometric measurements were averaged for each female prior to analysis to

prevent pseudoreplication. All variables were then tested for normality and homoscedasticity using Shapiro-Wilk and Levene tests respectively, and data were logarithm-transformed when necessary. Values reported are the mean ± s.e.m, unless otherwise stated. *P*-values and the associated test-statistic are reported for all tests in addition to the coefficient of determination (R^2), included for significant relationships to allow interpretation of variation explained by the model. Statistical significance was accepted if $P \leq 0.05$.

6.3 Results

General linear models were used to identify whether the physiological health of female freshwater turtles was related to reproductive investment. In each model, female haematological and biochemical values acted as independent variables while clutch size and egg morphometric measurements were dependent variables. Results of the general linear models are presented for each species in Tables 6.1 – 6.3. Parasitaemia was not detected in *E. macquarii* so this variable was disregarded during statistical investigations for this species.

An increase in WBC and H/L ratio was significantly related to the production of wider ($F = 2.75$, $P = 0.03$, $R^2 = 0.5192$) and heavier ($F = 9.65$, $P < 0.01$, $R^2 = 0.9301$) eggs respectively, for *E. macquarii*, although an elevated H/L ratio in this species was also associated with a decrease in egg width ($F = 10.09$, $P < 0.01$, $R^2 = 0.9357$) (Table 6.2). Similarly, raised PCV was also correlated with the production of narrower eggs in *E. macquarii* ($F = 2.86$, $P = 0.02$, $R^2 = 0.5385$; Table 6.2).

Female *C. oblonga* with elevated TP levels produced eggs that were heavier ($F = 2.58$, $P = 0.03$, $R^2 = 0.399$) and wider ($F = 2.46$, $P = 0.03$, $R^2 = 0.377$) than females of the same species with lower TP levels (Table 6.1). Similarly, *E. macquarii* females with increased TP produced larger clutches ($F = 3.96$, $P = 0.01$, $R^2 = 0.6909$) that consisted of longer eggs ($F = 3.01$, $P = 0.02$, $R^2 = 0.5649$; Table 6.2). Raised GLU levels also corresponded to a larger clutch size for *E. macquarii* ($F = 3.09$, $P = 0.02$, $R^2 = 0.5768$, Table 6.2) and *C. longicollis* ($F = 2.67$ $P = 0.04$, $R^2 = 0.5429$; Table 6.3), and although it was also significantly related to an increase in *E. macquarii* egg length ($F = 2.48$, $P = 0.04$, $R^2 = 0.4674$, Table 6.2) it was associated with a decrease in *C. oblonga* egg length ($F = 3.17$, $P = 0.01$, $R^2 = 0.5009$; Table 6.1). While an elevated A/G ratio was significantly related to an increase in clutch size ($F = 2.63$, $P = 0.04$, $R^2 = 0.5355$) for *C. longicollis* (Table 6.3), it also corresponded to a decrease in the mass ($F = 3.84$, $P < 0.01$, $R^2 = 0.5963$) and width ($F = 3.68$, $P < 0.01$, $R^2 = 0.575$) of *C. oblonga* eggs (Table 6.1). Increased AST was also significantly associated

with the mass of C. longicollis and *E. macquarii* eggs ($F = 3.12$, $P = 0.02$, $R^2 = 0.6192$ and $F = 2.44$, $P = 0.05$, $R^2 = 0.4603$ respectively) in addition to egg width ($F = 3.59$, $P = 0.01$, $R^2 = 0.6825$) of *C. longicollis* eggs (Table 6.3).

Female and egg morphometric measurements are reported in Table 6.4. Female BC was calculated from the linear regression of BM on CL and the resulting residual values represent both above (positive residual value) and below (negative residual value) average BC for each female. Multiple linear regression was used to compare CL and BC to CS and egg morphometrics (EM, EL and EW). Results of these tests are presented in Table 6.5 and show that female CL had a significant influence on reproductive investment for *C. oblonga* and *C. longicollis* in addition to BC for *C. oblonga*. Neither of these variables significantly affected reproductive investment in *E. macquarii* (Table 6.5). Larger *C. oblonga* and *C. longicollis* females produced bigger clutches ($T = 3.63$, $P < 0.01$, $R^2 = 0.5933$ and $T = 2.61$, $P = 0.05$, $R2 = 0.4143$ respectively) that consisted of heavier eggs ($T = 3.51$, $P < 0.01$, $R^2 = 0.4323$ and $T = 2.51$, $P = 0.05$, $R2 = 0.5602$ respectively). Larger *C. oblonga* also produced wider eggs ($T = 3.76$, $P < 0.01$, $R^2 = 0.4496$). Similarly, female *C. oblonga* in better condition also produced eggs that were heavier ($T = 2.68$, $P = 0.03$, $R^2 = 0.2522$) and wider ($T = 2.88$, $P = 0.02$, $R^2 = 0.2636$) than those in poorer condition (Table 6.5).

Additionally, ANCOVA was used to determine whether there was a trade-off between clutch size and egg morphometrics while controlling for female CL and BC. There was no significant relationship between the size of the clutch produced and the egg morphometrics for any species (Table 6.6).

Table 6.1. Results of generalised linear models identifying the relationship between female *Chelodina oblonga* physiological health indices and measures of reproductive investment including clutch size and mean egg mass, egg length and egg width. *Statistical significance was accepted if P ≤ 0.05.

Health Index	Clutch traits							
	Cutch size		Egg mass		Egg length		Egg width	
	T - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value
Haemoparasites	1.52	0.16	0.51	0.62	0.89	0.40	0.14	0.89
Haematocrit	1.05	0.32	0.36	0.73	0.03	0.97	0.48	0.64
Total white cell count	0.22	0.83	0.68	0.51	2.07	0.07	0.20	0.85
H/L ratio	1.03	0.33	0.14	0.89	0.35	0.74	0.19	0.86
Ca/P ratio	0.04	0.97	0.31	0.76	1.16	0.27	0.26	0.80
Aspartate transaminase	0.71	0.50	1.07	0.31	0.10	0.92	1.84	0.10
Creatine kinase	0.94	0.37	0.28	0.78	0.05	0.96	0.67	0.52
Glucose	0.38	0.71	1.63	0.13	3.17	**0.01***	0.99	0.35
Total protein	0.96	0.36	2.58	**0.03***	2.04	0.68	2.46	**0.03***
A/G ratio	1.06	0.31	3.84	**<0.01***	1.77	0.11	3.68	**<0.01***

Table 6.2. Results of generalised linear models identifying the relationship between female *Emydura macquarii* physiological health indices and measures of reproductive investment including clutch size and mean egg mass, egg length and egg width. *Statistical significance was accepted if P ≤ 0.05.

Health Index	Clutch traits							
	Cutch size		Egg mass		Egg length		Egg width	
	T - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value
Haemoparasites	-	-	-	-	-	-	-	-
Haematocrit	1.83	0.11	1.64	0.15	1.14	0.29	2.86	**0.02***
Total white cell count	0.31	0.77	2.03	0.08	1.55	0.17	2.75	**0.03***
H/L ratio	1.49	0.18	9.65	**<0.01***	0.91	0.40	10.09	**<0.01***
Ca/P ratio	1.45	0.19	0.64	0.54	0.24	0.82	1.44	0.19
Aspartate transaminase	1.76	0.12	0.99	0.35	0.27	0.80	2.44	**0.05***
Creatine kinase	0.06	0.95	0.35	0.74	1.32	0.23	1.30	0.24
Glucose	3.09	**0.02***	0.80	0.45	2.48	**0.04***	0.18	0.86
Total protein	3.96	**0.01***	2.00	0.09	3.01	**0.02***	1.21	0.27
A/G ratio	0.43	0.68	0.57	0.59	1.25	0.26	0.44	0.68

Table 6.3. Results of generalised linear models identifying the relationship between female *Chelodina longicollis* physiological health indices and measures of reproductive investment including clutch size and mean egg mass, egg length and egg width. *Statistical significance was accepted if $P \leq 0.05$.

Health Index	Clutch traits							
	Cutch size		Egg mass		Egg length		Egg width	
	T - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value	*T* - statistic	*P*- value
Haemoparasites	0.33	0.76	0.89	0.41	0.29	0.79	0.89	0.41
Haematocrit	1.04	0.34	0.60	0.57	0.24	0.82	0.67	0.53
Total white cell count	0.67	0.53	0.28	0.79	0.53	0.61	0.41	0.70
H/L ratio	0.41	0.70	0.03	0.97	1.91	0.11	0.23	0.83
Ca/P ratio	0.48	0.65	0.44	0.68	0.82	0.44	0.39	0.71
Aspartate transaminase	1.08	0.32	3.12	**0.02***	0.38	0.72	3.59	**0.01***
Creatine kinase	0.12	0.91	1.37	0.22	0.01	0.99	1.50	0.19
Glucose	2.67	**0.04***	1.30	0.24	0.56	0.60	1.46	0.20
Total protein	0.94	0.38	0.92	0.39	1.81	0.12	0.81	0.45
A/G ratio	2.63	**0.04***	0.94	0.38	0.17	0.87	1.01	0.35

Table 6.4. Clutch size in addition to female and egg morphometric measurements for three species of freshwater turtle (*Chelodina oblonga*, *Chelodina longicollis* and *Emydura macquarii*).

Measurement	*n*	Mean ± SE	Median	Range
Chelodina oblonga				
Female body mass (kg)	12	1.63 ± 0.03	1.63	0.86 - 2.14
Female carapace length (cm)	12	25.45 ± 0.18	25.00	21.90 - 28.50
Clutch size	12	13.33 ± 0.23	13.00	7.00 - 18.00
Egg mass (g)	138	10.78 ± 0.09	10.77	7.91 - 13.02
Egg length (mm)	138	34.10 ± 0.13	34.40	29.70 - 38.10
Egg width (mm)	138	22.74 ± 0.08	22.80	19.10 - 25.00
Emydura macquarii				
Female body mass (kg)	9	2.68 ± 0.04	2.64	1.94 - 3.20
Female carapace length (cm)	9	30.73 ± 0.25	29.10	26.20 - 34.90
Clutch size	9	22.80 ± 0.39	23.00	15.00 - 30.00
Egg mass (g)	167	9.86 ± 0.10	10.05	6.64 - 12.12
Egg length (mm)	167	35.43 ± 0.21	35.40	28.60 - 42.90
Egg width (mm)	167	21.46 ± 0.09	21.30	19.20 - 23.90
Chelodina longicollis				
Female body mass (kg)	8	0.92 ± 0.04	0.79	0.54 - 1.64
Female carapace length (cm)	8	21.68 ± 0.37	20.50	18.60 - 27.80
Clutch size	8	12.30 ± 0.52	11.00	6.00 - 18.00
Egg mass (g)	80	6.82 ± 0.18	6.02	5.17 - 10.26
Egg length (mm)	80	29.75 ± 0.18	29.60	26.10 - 32.70
Egg width (mm)	80	18.98 ± 0.21	18.35	15.80 - 23.30

Table 6.5. Results from multiple linear regressions used to compare maternal traits (carapace length and body condition) to clutch size and egg morphometrics (mean egg mass, length and width) for three species of freshwater turtle (*Chelodina oblonga*, *Chelodina longicollis* and *Emydura macquarii*).

		Maternal effects on clutch size and egg morphometrics					
		Chelodina oblonga		*Emydura macquarii*		*Chelodina longicollis*	
Maternal traits	Reproductive investment	*T*-statistic	*P*-value	*T*-statistic	*P*-value	*T*-statistic	*P*-value
Carapace length (cm)	Clutch size	3.63	**<0.01***	1.33	0.24	2.61	**0.05***
	Egg mass (g)	3.51	**<0.01***	0.75	0.49	2.39	0.06
	Egg length (mm)	2.05	0.07	1.44	0.21	0.84	0.44
	Egg width (mm)	3.76	**<0.01***	0.99	0.37	2.51	**0.05***
Body condition	Clutch size	0.14	0.89	0.12	0.24	2.11	0.09
	Egg mass (g)	2.68	**0.03***	0.30	0.78	0.64	0.55
	Egg length (mm)	1.15	0.28	0.76	0.48	0.80	0.46
	Egg width (mm)	2.88	**0.02***	0.38	0.72	0.79	0.47

Table 6.6. Results from an ANCOVA investigating the relationship between clutch size and egg morphometrics (mean egg mass, length and width) while controlling for maternal traits (carapace length and body condition) in three species of female freshwater turtle (*Chelodina oblonga*, *Chelodina longicollis* and *Emydura macquarii*).

		Effect on clutch size					
		Chelodina oblonga		*Emydura macquarii*		*Chelodina longicollis*	
Maternal traits	Egg morphometrics	*T*-statistic	*P*-value	*T*-statistic	*P*-value	*T*-statistic	*P*-value
Carapace length (cm)	Egg mass (g)	0.75	0.48	0.68	0.53	1.36	0.27
	Egg length (mm)	0.74	0.48	1.06	0.35	1.77	0.18
	Egg width (mm)	1.02	0.34	1.54	0.20	1.36	0.27
Body condition	Egg mass (g)	0.44	0.68	0.95	0.40	0.91	0.43
	Egg length (mm)	0.32	0.76	2.24	0.09	1.08	0.36
	Egg width (mm)	0.20	0.85	1.27	0.28	0.87	0.45

6.4 Discussion

Life-history theory predicts that because resources are finite, there is a trade-off in the allocation of these resources among different physiological systems (Roff 1992). As a result, females in poor condition may choose to invest in self-maintenance rather than reproduction (Gustafsson *et al.* 1994; Artacho *et al.* 2007a). Furthermore, optimum egg size theory, which is central to life history theory, then predicts that resources allocated to reproduction should endure a further trade-off between egg size and number in order to maximise maternal fitness (Smith & Fretwell 1974; Congdon & Gibbons 1987). Our results suggest that female turtles in poorer condition alter their level of reproductive investment. A decrease in reproductive investment was observed, manifested as changes in egg mass and size as well as clutch size, although different patterns were observed among species. Interestingly, female *E. macquarii* in better physiological state typically produced heavier and longer eggs than those in poorer state, whereas female *C. oblonga* and *C. longicollis* in better health invested in heavier and wider eggs. Furthermore, biochemical analyses of TP, GLU, A/G and AST generally correlated with numerous reproductive measures in each species, whereas haematological measures of PCV, WBC and H/L ratio were applicable only to *E. macquarii*. On the other hand, morphological measures of CL and BC were unrelated to reproductive investment in *E. macquarii*.

PCV is a measure of the efficiency of oxygen transport in the blood and a decrease in PCV is observed in anaemic individuals (Wagner *et al.* 2008). A significant relationship was identified between PCV and egg mass and width in *E. macquarii*. Although egg mass increased with PCV, egg width decreased, perhaps indicating that a trade-off exists between egg mass and width in this species. Perhaps healthier *E. macquarii* preferentially produce heavier rather than wider eggs in these instances. A decrease in PCV is regularly observed in female birds during clutch production, suggesting that investing energy in producing eggs compromises the blood-oxygen carrying capacity of the animal and potentially affects future reproductive events (Williams *et al.* 2004; Wagner *et al.* 2008). Clutch size was unrelated to PCV for all three species in this study, although further investigation is needed to identify whether females that produce more than one clutch trade-off future reproductive success by suffering a decrease in PCV during first clutch production.

WBC and H/L ratio represent immune system function and elevated measures of each suggest possible inflammation due to infection (Diethelm *et al.* 2006; Van Rijn & Reina 2010). A decrease

in reproductive investment in female dragons (*Ctenophorous fordi*) has been documented due to an increase in immune system function, apparent in decreased egg mass (Uller *et al.* 2006). Similarly, lower egg mass was also observed in eggs of *E. macquarii* in females with an elevated H/L ratio. Furthermore, elevated WBC resulted in an increase in egg width in this species, supporting the concept that healthier females trade-off egg width to produce heavier and longer eggs. Increased H/L ratio is often seen in female great tits during the reproductive process (Ots *et al.* 1998). Females typically have higher H/L ratios than males, presumably related to the stress of brood rearing and their higher level of parental investment (Kilgas *et al.* 2006; Norte *et al.* 2010).

Plasma proteins play a role in transport and immune system function and have proven to be extremely important indicators of health and nutritional state (Ots *et al.* 1998; Artacho *et al.* 2007a). Albumin and globulin are the two primary components of plasma protein and as well as acting as a metabolite carrier, albumin is an amino acid pool for protein synthesis and may also perform as an energy source (Ots *et al.* 1998), whereas globulin functions in assisting individuals to withstand the period immediately following injury (Ots *et al.* 1998). A decrease in TP over time is termed 'hypoproteinaemia' and is associated with many diseases, although most prevalent during instances of malnutrition (Ots *et al.* 1998). In our study, elevated TP concentrations were considered an indication of better health and were related to an increase in *E. macquarii* clutch size and egg length in addition to *C. oblonga* egg mass and width. Additionally, a higher A/G ratio was associated with the production of larger clutches for *C. longicollis*, but a decrease in the mass and width of *C. oblonga* eggs. Although higher A/G ratios are typically seen in healthier individuals (Kawai 1973), which would explain the increase in *C. longicollis* clutch size, they also indicate the under-production of globulin that may arise from excess manufacture of glucocorticoid. Glucocorticoid, or stress hormone, secretion is one of the most common responses during stress (Johnstone *et al.* 2012) and has been linked to reduced reproductive investment (Bonier *et al.* 2009). Therefore, it is plausible that the reduction in *C. oblonga* egg mass and width may be due to a stress related decrease in globulin production, although this is purely speculative and further research is needed.

Glucose is the principle metabolite resulting from carbohydrate metabolism, occurring as a direct result of food intake or from the metabolism of glycogen stores (Artacho *et al.* 2007a). Increased GLU concentration corresponded to increased clutch size in *E. macquarii* and *C. longicollis* in addition to the production of longer eggs by *E. macquarii*. This is likely the case because healthier females with a higher GLU are presumably obtaining more food and can invest more energy in

reproduction. Conversely, the length of *C. oblonga* eggs decreased as GLU increased, possibly because females of this species that are in better health choose to produce heavier and wider eggs instead. This trade-off in egg length and width opposes the tactic employed by *E. macquarii* and reinforces that species-specific trade-offs in reproductive investment exist. Maintenance of adequate GLU concentrations in blood plasma or serum is pivotal for the maintenance of nervous system function in birds (Rodriguez *et al.* 2005) and it also plays a pivotal role in preventing erythrocyte cryoinjury in reptiles during periods of sub-optimum temperature exposure (Costanzo *et al.* 1993).

An increase in AST and CK plasma enzyme concentrations can be indicative of cellular damage (Totzke *et al.* 1999). Plasma AST is not considered organ specific due to its presence and activity in many tissues, although increases in AST may be associated with impaired liver function (Boyd 1988; Campbell 1995). In order to distinguish between liver and muscle damage, the assessment of muscle-specific CK is generally also included in biochemical assessments of health in addition to AST (Dabbert & Powell 1993). During our study, CK had no significant effect on reproductive investment in any of the species ruling out muscle damage, although AST did in *C. longicollis* and *E. macquarii*, suggesting that impaired liver function in these females may have resulted in reduced reproductive investment. Female *C. longicollis* with increased AST produced eggs that were lighter and wider. Similarly, female *E. macquarii* with elevated AST produced lighter eggs. However, a complete analysis of liver function was not conducted during this study so it is difficult to determine whether this is actually the case. High AST levels have been recorded in black swan (*Cygnus melanocoryphus*) exhibiting liver damage as a result of heavy metal accumulation (Artacho *et al.* 2007a).

Larger female spotted turtles (*Clemmys guttata*) produce wider eggs than smaller turtles (Rasmussen & Litzgus 2010). Likewise, *C. oblonga* (measured by CL) produced eggs that were wider, and larger females of both *C. oblonga* and *C. longicollis* produced bigger clutches of heavier eggs. Furthermore, female *C. oblonga* in better BC also produced heavier and wider eggs. Investing in wider eggs does not result in the production of larger hatchlings (Rasmussen & Litzgus 2010), although investing in heavier and longer eggs does (Steyermark & Spotila 2001). However, a significant relationship between hatchling size and fitness remains to be identified (Congdon *et al.* 1999; Rasmussen & Litzgus 2010) and variation in egg size may be related to other factors such as the physical constraint imposed by female pelvis size (Rasmussen & Litzgus 2010). Nonetheless, the measure of CL and BC as biometric indicators of physiological health is questionable because studies

involving the western pond turtle (*Emys marmorata*) have identified that larger females with higher BC were actually in poorer physiological health than smaller females with a lower BC (Polo-Cavia *et al.* 2010), possibly because CL and BC vary intra-specifically as a result of differing levels of phenotypic plasticity to the local environment (Rowe 1997). Furthermore, morphometric indices are also unrelated to haematological and biochemical measures of health state in several species of chelonians (Scheelings & Rafferty 2012).

In summary, our results demonstrate that female freshwater turtles appear to alter clutch and/or egg size in accordance with physiological health state, agreeing with the physiological constraint hypothesis (Bowden *et al.* 2004). Additionally, no evidence of a trade-off between egg and clutch size existed during this study, refuting the optimum egg size hypothesis and agreeing with previous findings in other species of turtle (Wallace *et al.* 2007). This is possibly because females choose to produce as many eggs of sufficient size and quality as they can, given their limited resources during egg generation (Wallace *et al.* 2007). Moreover, it is evident that different species preferentially invest in different aspects of reproduction, although the consequences of this variation in reproductive investment remain to be elucidated. The findings of this study highlight the need to investigate the role that maternal health plays in dictating the reproductive success of a species. Focusing on improving the health of mothers in addition to increasing hatching success in waning populations may prove successful for the conservation and management of threatened and endangered species.

6.5 Acknowledgements

We thank the Holsworth Wildlife Foundation and Monash University for financial support in addition to the ABAXIS Company for generously donating the biochemistry rotors. This study was made possible with help from Bryan Tormey, Jason Van Rijn, Roger Evans, Zoos Victoria, and the staff of the Healesville Sanctuary Wildlife Health Centre. Research was conducted under scientific permits issued by the Victorian Department of Sustainability and the Environment (10005293), and the Western Australian Department of Environment and Conservation (SF007435; CE002893). It was approved by the Animal Ethics Committee of Monash University (BSCI/2009/28).

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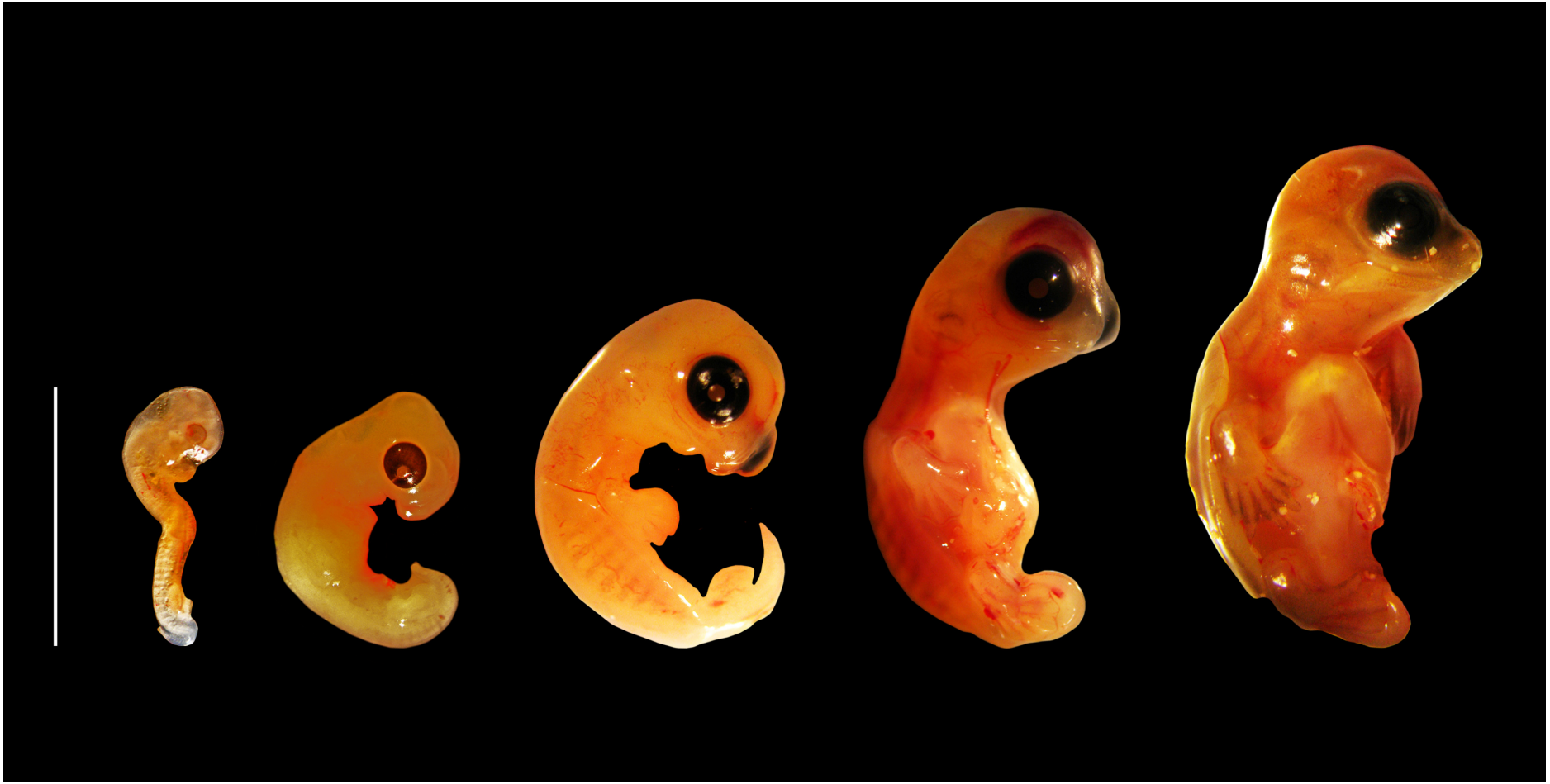
Top: Cathedral nets drying in the sun after retrieval
Bottom: A fyke net set at the edge of a shallow lake
Photos taken by Anthony R. Rafferty

Top: A funnel net attached to some reeds
Bottom: Retrieving an oblong turtle (*Chelodina oblonga*) from a funnel trap
Photos taken by Anthony R. Rafferty

Chapter Seven

General discussion and conclusions



Advancing stages of embryonic development in the Murray River turtle (*Emydura macquarii*). Scale bar represents 0.5 cm
Photos taken by Anthony R. Rafferty and compiled by Bryan P. Tormey

In this study I provided new data about how the environment that embryos experience both inside the mother and in the nest, influences development and directs the evolution of strategies to delay growth and hatching in reptile embryos. These results improve our understanding of reptilian reproductive strategies and inform us how animals have evolved beyond the limitations of oviparity. Additionally, they also highlight the adaptations that have arisen in response to the challenges associated with terrestrial invasion. The following sections provide a brief summary of the main findings presented in each chapter and discuss the general implications of these results.

7.1 Pre-ovipositional development

As previously discussed, the embryos of many lizard species and all turtles enter a state of pre-ovipositional developmental arrest *in utero* during periods of extended egg retention beyond that normally observed for the species (Ewert 1985; Miller 1985; Andrews & Mathies 2000; Rafferty & Reina 2012a). However, the mechanisms involved in this process needed to be fully investigated. In this thesis I demonstrated that the restricted diffusive ability of oviducal secretions creates or contributes to an extremely hypoxic oviducal environment, regulating the onset and maintenance of pre-ovipositional arrest in turtles (Rafferty *et al.* 2012a). First, the extremely low mean oviduct PO_2 was consistent across all four turtle species and was substantially more hypoxic than previous estimates in sceloporine lizards (Parker & Andrews 2006). Second, eggs that were transferred immediately at oviposition to a hypoxic incubation environment remained arrested until subsequent exposure to normoxia. This is similar to the process observed in natural nests of the north Australian long necked turtle (*Chelodina rugosa*) that lay eggs underwater in flooded billabongs, in which eggs do not begin developing until the nests dry out and O_2 tension increases (Kennett *et al.* 1993). Finally, oviducal secretions significantly retarded O_2 diffusion and may therefore be the primary factor involved in controlling oxygen availability within the oviduct of oviparous amniote species. Very little is known about the role that oviducal secretions play during the reproductive process but it has been hypothesized that they may possess anti-microbial properties that protect the eggs from bacterial infection during and after oviposition (Hayssen & Blackburn 1985). However, based on observations not presented in this thesis, I do not believe this to be the case and instead, I consider the oviducal secretions to play an important role in regulating the oxygen environment in the reptile oviduct and perhaps aid oviposition by also acting as a lubricant.

Additionally, my investigations suggest that periods of extended arrested development can negatively affect embryo survival (Rafferty *et al.* 2011). In the leatherback turtle (*Dermochelys coriacea*), the likelihood that embryos would fail to recommence development after oviposition increased with the duration spent in arrest, a phenomenon also observed in *C. oblonga* and *C. mydas* embryos (Rafferty & Reina 2012b). It is plausible that increased mortality in these latter laboratory tests was due to artificial manipulation of the incubation environment because no significant difference was observed in the proportion of eggs dying in the normoxic or hypoxic treatments for *C. longicollis* or *E. macquarii* even though they were incubated under the same conditions as the two other species (Rafferty & Reina 2012b).

Alternatively, it is also possible that the embryos of some species have needed to evolve the capability to withstand hypoxia and extended periods of arrest better than others. For example, the olive ridley marine turtle (*Lepidochelys olivacea*) can retain eggs for up to 63 days during prolonged internesting periods without any apparent affect on hatching success (Plotkin *et al.* 1997). The embryos of the olive ridley turtle are presumably better adapted to these long periods spent in the oviducts than species such as the leatherback turtle which typically only have short internesting periods, laying eggs every one to two weeks (Rafferty *et al.* 2011). However, this latter suggestion assumes that the hypoxia and / or the arrest are stressful to the embryo, resulting in a negative impact on survival if embryos are not capable of withstanding the pressure exerted upon them. This could possibly be a mechanism that ensures only the fittest embryos survive or perhaps a method by which mothers 'condition' their embryos in some way to withstand challenges experienced at a later stage during development, as seen in *C. elegans* embryos that become 'conditioned' to withstand hypoxia (Miller & Roth 2009). Although this is purely speculative, it is evident that a considerable amount of work is needed to fully understand the process of pre-ovipositional arrest and the impact that it has on embryo development and survival.

7.2 Post-ovipositional development

My results show that the time taken to break pre-ovipositional arrest after oviposition was independent of incubation temperature (Rafferty & Reina 2012b), which is surprising considering that reptilian development is positively correlated with temperature during earlier rather than later stages (Webb 1987; Andrews 2004). Furthermore, in the low temperature treatment, active embryonic development recommenced in all species except *C. oblonga* despite an incubation

temperature that was too low to facilitate successful embryonic development through to hatching. Surely, recommencing development at sub-optimum incubation temperatures is disadvantageous for the embryo if the temperature fails to subsequently increase, suggesting that embryos may be hedging their bets that this temperature increase is sure to occur. Alternatively, perhaps the embryos are incapable of actually 'choosing' when to recommence development, doing so regardless of the immediate incubation environment. In this instance, the mother must select the most appropriate time to nest that ensures her embryos have the greatest chance of survival. Consequently, early stage developmental success may be dictated by the mother rather than the embryos themselves (Rafferty *et al.* 2011) and cold torpor, as seen in *C. oblonga* embryos in the low temperature treatment, may be a back-up plan for the embryo if mothers do not nest at the most opportune time.

Although post-ovipositional embryonic diapause was not observed during my investigations, *E. macquarii* and *C. mydas* embryos appeared to down-regulate development and delay hatching, which subsequently resulted in embryo mortality probably due the absence of a thermal stimulus to pip (Rafferty & Reina 2012b). Presumably, a temperature change was required to stimulate pipping and hatching in both of these species This has been proposed previously for *E. macquarii* (Thompson 1989; Ewert 1991), but I present the first evidence in *C. mydas*, indicating that sea turtles possibly retain remnant traces of an ability they once possessed, or perhaps that they may be pre-adapted to evolve delayed hatching in the future (Ewert 1991; Rafferty & Reina 2012b). This finding may have implications for the conservation and management of threatened and / or endangered sea turtle species, suggesting that eggs developing in nests laid late in the season that are experiencing temperatures thought to be too low to facilitate development, may actually be successfully completing development and just need to be removed and stimulated to hatch. Interestingly, *E. macquarii* embryos are also capable of increasing their developmental rate close to hatching so that underdeveloped embryos can "catch up" with more developed siblings and hatch together (Thompson 1989; McGlashan *et al.* 2011). Clearly, some turtle species possess a diverse array of strategies to alter their developmental rate so that pipping and hatching occur when conditions are most conducive to survival. It is therefore plausible that a spectrum exists in which embryos are capable of either hatching early or late in order to optimise their chance of success.

7.3 Maternal reproductive investment

Offspring investment in oviparous reptiles is generally restricted to pre-ovipositional allocations to egg size and number, because females do not usually provide parental care after they lay their eggs. Therefore, it is extremely important to fully understand how reptile mothers invest in reproduction and the success of their young and it is apparent from my investigations that a considerable amount of work remains to be done on this topic. As previously stated, life-history theory predicts that a trade-off in the allocation of resources among different physiological systems exists because resources are finite, and as a result, females in poor health may choose to invest in self-maintenance rather than reproduction. My results suggest that female turtles in poorer condition decrease their level of reproductive investment, manifested by changes in egg mass and size as well as clutch size, depending on species. (Rafferty *et al.* 2012b; Scheelings & Rafferty 2012). Interestingly, maternal effects have also been linked to other aspects of reproduction and development in turtles including the occurrence and duration of several types of developmental arrest, hatching success, hatchling growth rate and righting response (Ewert 1991; Steyermark & Spotila 2001a; Steyermark & Spotila 2001b; Booth 2002), highlighting the connection between life-history evolution and female physiology. Additionally, I also demonstrated in this thesis that some female leatherback turtles are more successful mothers than others (Bell *et al.* 2004; Rafferty *et al.* 2011). Collectively, all of my findings highlight the need to investigate the role that mothers plays in dictating the success of their young. Focusing on improving of our understanding of maternal investment in addition to embryonic development, may prove successful for the conservation and management of threatened and endangered species in waning populations. It will also contribute to our intellectual understanding of life history theory and the evolution of different reproductive modes.

7.4 Conservation and management implications

The development and implementation of successful biological management programs require an understanding of the reproductive strategies used by a species, and comparative studies like this one therefore have considerable conservation implications. Conducting such investigations allow (1) the generation of captive breeding programs that take into account the developmental biology associated with each species in order to accurately replicate the natural daily and seasonal cycles; (2) recognition of the impacts that increased global temperature associated with climate change is likely to have on developmental success; (3) offer further insights into the evolutionary

relationships among reptile species residing in different orders; (4) provide a basis for medical and veterinary researchers to develop treatments to slow or arrest rapid cell division; and (5) in the case of this study, afford collaboration with local indigenous land management groups and government bodies to ensure protection and conservation of natural Australian fauna. Additionally, understanding what induces a state of early stage embryonic arrest may allow the successful transport of eggs without risk of movement induced mortality, thus allowing for ex-situ research on a multitude of different species, particularly those that are protected and/or endangered. Finally, this work offers a fundamental understanding of the diversity of reptilian developmental processes and their consequences.

7.5 Study scope and directions for future research

The purpose of this section is to highlight the limitations of this study and in doing so, provide direction for future research and build on that previously discussed in section 2.4 of this thesis. The scope of this study was a single representative order within the Class Reptilia. Therefore, a comparative approach that investigates the reproductive strategies employed by various different reptile taxa would be beneficial, particularly with regard to understanding the evolutionary transition between reproductive modes. For example, in several squamates, the simultaneous occurrence of both oviparity and viviparity in different populations of the same species has been observed and offers the opportunity to identify how the maternal environment impacts on the degree of oviducal embryonic development.

The effect that oxygen availability has on embryonic development *in utero* is fascinating and there is room for a substantial amount of future investigation in this area. For instance, my research did not identify when oviducal hypoxia arises during development and it would be useful to measure oviducal PO_2 over time beginning immediately after fertilisation, to ascertain whether oviducal PO_2 varies in relation to the stage of embryonic development. Additionally, pre-ovipositional arrest is generally observed during early embryogenesis but there are species in which it arises late in development. It would be interesting to measure and compare the degree of embryonic development that corresponds with oviducal PO_2 in these species. We predict that the oviducal PO_2 of oviparous lizards that arrest embryonic development late in the developmental schedule will be substantially higher (to meet the embryonic oxygen demand to facilitate advanced development) than that of turtles or other species that arrest embryonic development during early stages. Further, measuring PO_2 at several locations along the oviduct would identify whether

embryos are exposed to different PO_2 levels as they pass down the oviduct (moving from the ovary after ovulation towards the cloaca for oviposition). It may also be beneficial to conduct a comparative histological analysis of the oviduct lining of species that arrest at different stages of development. Perhaps species that arrest during early stages of development have less uterine vascularisation than species that arrest during later stages of embryogenesis, due to the lower relative oxygen demand of embryos in the oviduct.

More work is needed to identify the role that oviducal secretions play in reproduction and development in oviparous species. For example, are these secretions produced in utero at specific stages during embryonic development to create a hypoxic environment and arrest development? Additionally, do species that do not use pre-ovipositional arrest produce these secretions and do they have similar properties? Perhaps a histological or molecular study that involves analysing the uterine tissue of gravid females when embryos are at various stages of development will help to identify when and how these secretions are produced and clarify the role that they play during reproduction.

Finally, this study primarily used experimental conditions in a laboratory environment to study development within the egg. However, a study approach that incorporates field investigations and monitors development and growth of hatchlings after eggs have hatched will validate the laboratory-based data and thereby give us a firmer understanding of how maternal and nest factors affect offspring survival.

7.6 Concluding remark

The findings presented in this thesis contribute to our understanding of how ecology shapes the evolution of developmental processes observed in reptile embryos, in addition to providing evidence of the mechanisms underlying the evolutionary transition between reproductive modes. It sets the foundation for future research, particularly investigations that focus on understanding how maternal effects influence embryonic development both before and after oviposition in reptiles, despite the absence of parental care in many species.

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Heron Island, Australia
Photo taken by T. Franciscus Scheelings

Appendix A

Hematologic and serum biochemical values of gravid freshwater Australian Chelonians

T. Franciscus Scheelings[1] & Anthony R. Rafferty[2]

[1]Australian Wildlife Health Centre, Healesville Sanctuary, Healesville, Victoria, Australia

[2]Australian Centre for Biodiversity, School of Biological Sciences, Monash University, Melbourne, Victoria, Australia

Published under the same name in Journal of Wildlife Diseases

Published 4th April 2012

Declaration for Appendix A

Monash University

Declaration by candidate

In the case of Appendix A, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Ethics, permits, data collection and analysis, manuscript editing	50%

The following co-author contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
T. Franciscus Scheelings	Data collection and manuscript preparation	50%

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	Monash University, School of Biological Science, Clayton campus

	Signature	Date
T. Franciscus Scheelings		09/05/2012

Drawing blood from the jugular vein on an oblong turtle (*Chelodina oblonga*)
Photo taken by Anthony R. Rafferty

Journal of Wildlife Diseases, 48(2), 2012, pp. 314–321

HEMATOLOGIC AND SERUM BIOCHEMICAL VALUES OF GRAVID FRESHWATER AUSTRALIAN CHELONIANS

T. Franciscus Scheelings,[1,3] and Anthony R. Rafferty[2]

[1] Australian Wildlife Health Centre, Healesville Sanctuary, Badger Creek Road, Healesville, Victoria, 3777, Australia
[2] School of Biological Sciences, Monash University, Wellington Road, Clayton, Victoria, 3800, Australia
[3] Corresponding author (email: fscheelings@zoo.org.au)

ABSTRACT: Hematologic and serum biochemical analyses were performed on 30 wild-caught, gravid, Australian freshwater chelonians. Species sampled were western long-necked turtles (*Chelodina oblonga*; $n=13$), common long-necked turtles (*Chelodina longicollis*; $n=8$), and Murray River turtles (*Emydura macquarii*; $n=9$). Turtles were obtained from Lake Goolellal in Perth, Western Australia (*C. oblonga*), and Lake Coranderrk in Healesville, Victoria (*C. longicollis* and *E. macquarii*). All turtles were considered healthy at the time of sample collection. Blood results were similar to those reported in other freshwater chelonians, with the exception of elevated calcium levels in all species. Hypercalcemia was attributed to egg development and maturation. A hemoparasite morphologically resembling *Haemogregarina clelandi* was found in all *C. oblonga* samples and in four *C. longicollis* samples. Infection with *H. clelandi* appeared to have no physiological effects on blood parameters or morphometrics of infected turtles. Blood parameters were also considered poor indicators of female chelonian morphometrics and fecundity.

Key words: Biochemistry, *Chelodina longicollis*, *Chelodina oblonga*, common long-necked turtle, *Emydura macquarii*, hematology, Murray River turtle, western long-necked turtle.

INTRODUCTION

Hematologic and serum or plasma biochemical analyses are important in the clinical assessment of reptiles, although their usefulness depends on validated species-specific reference ranges. One of the challenges involved in using blood parameters to assess the health of animals is to remove sources of inherent and confounding variation potentially arising from factors relating to the individual animal. For example, individuals vary in age (or size), body condition, parasite load, sex and nutritional status, and such factors may influence hematologic and serum or plasma biochemical parameters (Harr et al., 2001; Campbell and Ellis, 2007; Hidalgo-Vila et al., 2007).

Side-necked turtles of the suborder Pleurodira comprise two families, Pelomedusidae and Cheluidae (Seddon et al., 1997). Excluding the unique pig-nosed turtle (*Carettochelys insulpta*), all Australian freshwater chelonians are members of the family Cheluidae, which is restricted to Australia, New Guinea, and South America (Seddon et al., 1997; Wilson and Swan, 2008). With the exception of Anderson et al. (1997), there are few comparative data on the hematology or biochemistry of the suborder Pleurodira. Endemic freshwater chelonians are commonly kept either as pets or display animals in zoological institutions in Australia. Gravid, freshwater chelonians are frequently presented to the Australian Wildlife Health Centre (Healesville Sanctuary, Victoria, Australia) for management of traumatic injuries and their triage is hampered by a lack of normal physiologic data.

Our aims were to obtain basic morphometric data of gravid, wild Australian freshwater chelonians, establish preliminary baseline values for hematological and serum biochemical parameters in these species, determine the prevalence of hemoparasites, and determine if blood values and hemoparasite load can be used as predictors of fecundity or morphology.

MATERIALS AND METHODS

Animals

Thirty, wild, gravid, freshwater Australian chelonians were captured for assessment of hematologic and biochemical values. Species

that were included in this study were western long-necked turtles (*Chelodina oblonga*; $n=13$), common long-necked turtles (*Chelodina longicollis*; $n=8$), and Murray River turtles (*Emydura macquarii*; $n=9$). *Chelodina oblonga* were trapped at Lake Goolellal (31°48′29″S, 115°47′38″E) in Perth, Western Australia, 1–7 October 2010 (spring) with the use of baited, modified funnel traps. In October 2010 the mean minimum temperature in Perth was 10.7 C and the mean maximum temperature was 24.6 C. During this time Perth experienced rainfall on five occasions with a maximum precipitation of 8.4 mm and total precipitation of 20.6 mm. No rainfall was recorded during trapping dates (Australian Government Bureau of Meteorology, 2011). Captured *C. oblonga* were housed in plastic tubs with moist towels and flown to Monash University, where they were transferred into large aquariums. All *C. oblonga* were sampled within 3 days of capture at the Australian Wildlife Health Centre.

Chelodina longicollis and *E. macquarii* were trapped at Lake Coranderrk (37°40′56″S 145°31′54″E) in Healesville, Victoria, between 10 October and 15 December 2010 (spring to early summer), with the use of baited fyke nets. During this time the mean minimum temperature in Healesville was 9.2 C and the mean maximum temperature was 22.4 C. From October–December 2010, Healesville experienced rainfall on 41 occasions, with a maximum precipitation of 58.4 mm and a total precipitation of 386.2 mm (Australian Government Bureau of Meteorology, 2011). Once captured, both species were immediately transferred to the Australian Wildlife Hospital (approximately 1 km from capture site) for blood collection. Only animals considered healthy by clinical examination were included in this study.

This investigation was conducted in concert with another project investigating egg development in female chelonians and was approved by the Monash University School of Biological Sciences Ethics Committee (BSCI/2009/28). Collection of chelonians was approved with permits obtained from the Victorian Department of Sustainability and the Environment. All animals were released alive at their point of origin at the conclusion of this investigation.

Sample collection and processing

The sample size for this investigation was restricted to the number of gravid females that could be captured within the time constraints of the ethics and collection permits. Nevertheless, the sample size correlated with previously published literature documenting the relationship between the physiological condition of female western pond turtles in California ($n=14$) and various aspects of female morphometrics (Polo-Cavia, 2010). Significant hematologic and biochemical relationships have also been detected in studies with sample sizes of as few as six individuals (Ots et al., 1998). It is therefore unlikely that significant relationships went undetected in this study.

Following capture, all animals were transported to the Australian Wildlife Health Centre so that sampling could be performed by an experienced reptile veterinarian. The presence of eggs was confirmed by radiography and chelonians were weighed, and carapace length and width measured. They were then manually restrained and 2 ml of blood was collected from the jugular vein with a 22-gauge needle attached to a 3-ml syringe. Half of the blood collected was immediately transferred into a lithium heparin container (BD Microtainer™ Tubes, Vacutainer Systems, Franklin Lakes, New Jersey, USA) and the other half into a plain container (BD Microtainer™ Tubes, Vacutainer Systems). A small volume of blood was also collected into microhematocrit tubes (Iris Sample Processing, Westwood, Massachusetts, USA). Blood in the plain tube was immediately centrifuged and the resulting serum analyzed with the use of the avian–reptilian rotor on the Vet Scan analyzer (Abaxis Inc., Union City, California, USA).

The packed cell volume (PCV) was determined with the use of standard centrifugation of the microhematocrit tubes. A leukocyte differential count was performed by examination of air-dried, whole-blood films on a microscope slide stained with Romanowsky stain (Rapid Diff, Australian Biostain Pty. Ltd., Traralgon, Victoria, Australia). Smears were made immediately after collection by dragging a droplet of blood across a glass microscope slide with a second glass slide. Leukocytes were classified as heterophils, lymphocytes, eosinophils, basophils, or monocytes. Heterophil/eosinophil counts were performed manually with a hemocytometer and an Avian Leukopet™ (Avian Leukopet™, Vetlab Supply, Palmetto Bay, Florida, USA) designed for counting eosinophils and heterophils. 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).

Blood smears were also used to identify and assess the prevalence of hemoparasites within

erythrocytes by examining 1,000 erythrocytes under 1,000× magnification and determining the percentage of infected cells. We considered this an estimate of parasite load within these species.

Statistical analysis

Data analyses were conducted with the use of R, statistical package 2.11.0 (R Development Core Team, 2010). Data were normally distributed and linear mixed models using the "LME" function in the 'nlme' package of R were used to determine if female body morphometrics (weight, carapace length, and carapace width) and prevalence of hemoparasites were predicted by hematologic and biochemical values, and whether these values differed among females of the same species. In the mixed models, female body morphometrics and hemoparasite count acted as response variables, the hematologic and biochemical values were the fixed effects, and the female individual identification number was the random effect. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were also conducted to identify whether there was a significant difference in hematologic and biochemical values between species. Degrees of freedom (df) and F, P, and r^2 values are included for all tests. Statistical significance was accepted if $P \leq 0.05$, and only r^2 results that showed a strong correlation have been reported.

RESULTS

The morphometric data, and hematologic and biochemical results for three species of gravid, freshwater, Australian chelonians are presented in Table 1. The PCV ranged from 17% to 35% in *C. oblonga*, from 16% to 26% in *C. longicollis*, and from 18% to 30% in *E. macquarii*. Erythrocyte morphology for all species was typical of that previously described in chelonians (Campbell and Ellis, 2007). Immature erythrocytes comprised <1% of the erythrocyte population. Thrombocytes had a darkly staining nucleus with a lightly staining cell membrane and colorless cytoplasm. Clumping prevented estimation of thrombocyte numbers in all three species.

The TWBC ranged from 6×10^3 to 16.9×10^3 cells/μl in *C. oblonga*, from 6×10^9 to 35.6×10^9 cells/l in *C. longicollis*, and from 2.4×10^3 to 9.5×10^3 cells/μl in *E. macquarii*. Leukocyte morphology was typical of that previously described in chelonians (Campbell and Ellis, 2007). The differences in TWBC observed between *C. longicollis* and *E. macquarii* was statistically significant (df=2, $F=7.92$, $P=0.002$). No statistically significant differences were found between the TWBC of *C. longicollis* and *C. oblonga* or *C. oblonga* and *E. macquarii*.

Percentage of heterophils was significantly greater in *C. oblonga* than *C. longicollis* (df=2, $F=7.14$, $P=0.0034$) but there was no significant difference detected between *E. macquarii* and *C. longicollis* or between *C. oblonga* and *E. macquarii*. Percentage of monocytes was significantly greater in *C. oblonga* than *C. longicollis* and *E. macquarii* (df=2, $F=9.62$, $P<0.001$) but no statistical difference was detected between *C. longicollis* and *E. macquarii*. Percentage of eosinophils was significantly greater in *C. longicollis* than in *C. oblonga* (df=2, $F=14.31$, $P<0.040$); however, no significant difference was detected between *E. macquarii* and *C. longicollis* or between *E. macquarii* and *C. oblonga*.

Between 0.1% and 0.9% of erythrocytes in *C. oblonga* and between 0% and 0.2% of erythrocytes in *C. longicollis* contained large (12×5 μm) sausage-shaped inclusions that had a dark-staining nucleus at one pole (Fig. 1). These inclusions were morphologically similar to hemoparasites previously identified as *Haemogregarina clelandi* (Mackerras, 1961; Jakes et al., 2001). The presence of intraerythrocytic hemoparasites resulted in enlargement of the infected RBCs and terminal displacement of their nuclei. No hemoparasites were observed in any blood smears from *E. macquarii* and a statistically significant difference in parasite number was detected among species (df=2, $F=14.63$, $P<0.001$). Hemoparasite numbers were not significantly correlated with PCV, serum globulin, serum protein, or female

TABLE 1. Morphometric, hematologic and serum biochemical values for wild-caught, gravid, western long-necked turtles (*Chelodina oblonga*; $n=13$), common long-necked turtles (*Chelodina longicollis*; $n=8$), and Murray River turtles (*Emydura macquarii*; $n=9$). *Chelodina oblonga* were captured from Lake Goolellal in Perth, Western Australia in October 2010. *Chelodina longicollis* and *E. macquarii* were captured from Lake Coranderrk in Healesville, Victoria between October and December 2010.

	C. oblonga		*C. longicollis*		*E. macquarii*	
Analyte (units)	Mean (SD)	Range (median)	Mean (SD)	Range (median)	Mean (SD)	Range (median)
Weight (gm)	1572 (360.4)	859.3–2,135.1 (1,597.8)	814.4 (351.7)	540–1,637.9 (705.1)	2,644.4 (515.6)	1,940–3,200 (2,640)
Carapace length (cm)	25.2 (2.1)	21.9–28.5 (24.9)	20.9 (3)	18.6–27.8 (19.9)	30.5 (3.3)	26.2–34.9 (29.1)
Carapace width (cm)	16.9 (2.76)	14.1–24 (15.9)	17 (1.7)	15.3–20.2 (16.3)	23.6 (3.6)	18.7–27.9 (25.7)
Eggs (*n*)	12.7 (2.87)	7–18 (12)	10.6 (4.6)	6–18 (9.5)	21.2 (5.2)	15–30 (25.7)
Packed cell volume (%)	28 (6)	17–35 (30)	22 (3)	16–26 (20)	24 (4)	18–30 (24)
Total white blood cells ($\times 10^9$/µl)	12.1 (3.76)	6–16.9 (12.7)	14.6 (9.4)	6–35.9 (12.7)	6.5 (2.1)	2.4–9.5 (6.9)
Heterophils (%)	53.9 (13.2)	36–77 (51.5)	28.1 (15)	9–58 (25)	45.9 (17.2)	18–69 (45)
Lymphocytes (%)	24.1 (7.9)	11–35 (23.5)	52.7 (18.4)	29–82 (51.5)	39.2 (19.4)	12–63 (49)
Monocytes (%)	18.4 (6.6)	6–27 (19.5)	9.1 (5.8)	2–19 (8)	7.8 (5.5)	1–18 (5)
Eosinophils (%)	3.1 (2.5)	0–7 (3)	9.1 (5.7)	4–20 (7)	6.9 (6.8)	1–24 (5)
Basophils (%)	0.5 (0.7)	0–2 (0)	0.9 (1.4)	0–4 (0.5)	0.2 (0.4)	0–1 (0)
Hemoparasites (*n*/1,000 RBC)	3.8 (2.56)	1–9 (3)	0.6 (0.7)	0–2 (0.5)	0 (0)	0 (0)
Aspartate aminotransferase (IU/l)	461.8 (199)	212–861 (466)	69.4 (21.8)	25–91 (72.5)	97.4 (39)	54–181 (92)
Creatinine kinase (IU/l)	2,608.6 (1,705.8)	259–5,244 (2,091)	126.9 (148.6)	17–390 (61.5)	568.7 (462.1)	100–1,360 (534)
Uric acid (mmol/l)	67.3 (26.7)	29–124 (69)	65.6 (18.6)	39–89 (72.5)	56.6 (12.3)	42–79 (54)
Glucose (mmol/l)	7.16 (2.9)	1.4–11.8 (7.15)	7.7 (1.6)	5–10.4 (7.8)	6.14 (1.6)	3.5–8.4 (5.9)
Calcium (mmol/l)	4.66 (0.4)	3.95–5 (4.7)	4 (0.5)	3.3–5 (3.9)	3.4 (0.8)	2–4 (4)
Phosphorus (mmol/l)	2 (0.5)	1.4–2.8 (2)	1.5 (0.3)	0.9–1.8 (1.5)	1.8 (0.6)	1.1–2.8 (1.6)
Total protein (g/l)	68 (4.9)	60–76 (68)	45 (4.6)	37–53 (1.5)	35 (5.7)	25–42 (36)
Albumin (g/l)	20 (2)	16–24 (21)	18 (1.8)	14–20 (18)	18 (3.3)	12–22 (18)
Globulin (g/l)	48 (4.4)	41–54 (48)	27 (4.1)	23–35 (26)	15 (6.9)	0–23 (17)
Potassium (mmol/l)	5.1 (0.9)	3.5–6.9 (4.9)	3.4 (0.5)	2.9–4.3 (3.3)	3.4 (0.4)	2.8–4.2 (3.4)
Sodium (mmol/)	131.5 (5.7)	126–147 (132)	130.2 (3.7)	124–136 (131)	131.1 (3)	128–135 (131)

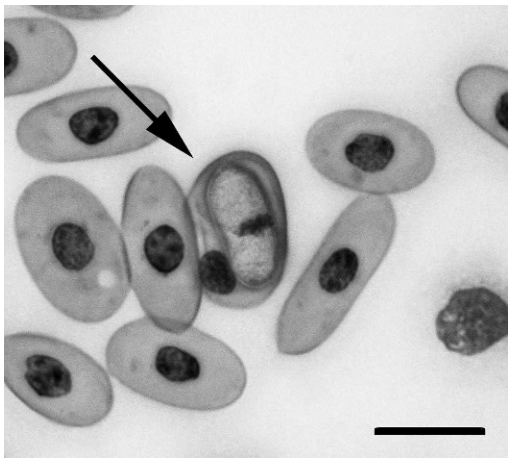

FIGURE 1. Erythrocyte from western long-necked turtle (*Chelodina oblonga*) captured from Lake Goolellal in Perth, Western Australia in October 2010, infected with hemoparasite suspected to be *Haemogregarina clelandi* (arrow). Romanowski stain. Bar=10 μm.

morphometrics (weight, carapace length, carapace width, or fecundity) in any species examined. However, TWBC was negatively associated with parasite count in *C. oblonga* (df=10, F=5.80, P=0.0369, r^2=0.37). A significant increase in percentage of circulating eosinophils was observed in *C. longicollis* as intraerythrocytic parasite numbers increased (df=6, F=11.86, P=0.0137, r^2=0.66).

Aspartate aminotransferase (AST) was significantly greater in *C. oblonga* than in *C. longicollis* or *E. macquarii* (df=2, F=28.98, P<0.001) but no significant difference existed between *C. longicollis* and *E. macquarii*. Creatinine kinase (CK) was greatest in *C. oblonga* (df=2, F=13.96, P<0.001) but no difference was detected between *C. longicollis* and *E. macquarii*. Calcium (Ca^{2+}) was also greatest in *C. oblonga* (df=2, F=12.44, P<0.001) but no difference was detected between *C. longicollis* and *E. macquarii*. Total protein (TP) differed significantly between all species (df=2, F=114.46, P<0.001) and was highest in *C. oblonga* and lowest in *E. macquarii*. Albumin (Alb) was significantly higher in *C. oblonga* than in *E. macquarii* (df=2, F=3.92, P=0.0325) but there were no differences detected between *E. macquarii* and *C. longicollis* or between *C. oblonga* and *C. longicollis*. Globulin (Glob) differed significantly among species (df=2, F=103.04, P<0.001) and was highest in *C. oblonga* and lowest in *E. macquarii*. Potassium (K^+) was significantly higher in *C. oblonga* (df=2, F=19.59, P<0.001), but no differences existed between *C. longicollis* and *E. macquarii*. Bile acids could not be measured in any species with the use of the Abaxis rotor.

DISCUSSION

For all species examined, RBC and leukocyte morphology were similar to those described in other chelonians (Campbell and Ellis, 2007; Chung et al., 2009; Chansue et al., 2011). Packed cell volume was not significantly different between *C. longicollis* and *E. macquarii* and was similar to values reported in other chelonians (Diethelm and Stein, 2006; Hidalgo-Vila et al., 2007; Innis et al., 2007; Perpiñán et al., 2008; Chung et al., 2009; Chansue et al., 2011). Greater total protein in *C. longicollis* than in *E. macquarii* is most likely due to diet. *Chelodina longicollis* is an obligate carnivore (Chessman, 1984), whereas *E. macquarii* is an omnivore with filamentous algae constituting approximately 53% of the diet (Spencer et al., 1998). The higher PCV of *C. oblonga* compared *C. longicollis* and *E. macquarii* was still within normal limits of other freshwater turtles (Rangel-Mendoza et al., 2009; Rossini et al., 2010), although TP was higher than in other species of aquatic chelonians (Anderson et al., 1997; Diethelm and Stein, 2006; Hidalgo-Vila et al., 2007; Innis et al., 2007; Perpiñán et al., 2008; Chung et al., 2009; Rangel-Mendoza et al., 2009; Rossini et al., 2010; Chansue et al., 2011). The increased erythrocyte volume and serum protein in *C. oblonga* may be normal in this species or it could possibly reflect mild dehydration at the time of sampling

due to transportation. None of the *C. oblonga* showed clinical signs of dehydration when they were initially examined, but this may be difficult to detect in some species of reptiles and thus only be apparent with biochemical analysis.

The TWBC and leukocyte differentials for all three species examined were similar to those of other freshwater chelonians (Hidalgo-Vila et al., 2007; Chung et al., 2009; Rangel-Mendoza et al., 2009; Rossini et al., 2010; Chansue et al., 2011). Variation in the leukocyte differential counts observed between species is likely to reflect species-specific variation. Leukocyte counts in reptiles may be influenced by age (or size), body condition, parasite load, sex, environmental conditions and nutrition and these need to be considered carefully when interpreting results (Harr et al., 2001; Campbell and Ellis, 2007; Hidalgo-Vila et al., 2007; Rangel-Mendoza et al., 2009).

A hemoparasite morphologically resembling *Haemogregarina clelandi* was found in all 13 blood samples from *C. oblonga* and in four blood samples from *C. longicollis*. This parasite was not seen in any blood smears from *E. macquarii*, despite these animals originating from the same water body as *C. longicollis*. *Haemogregarina clelandi* has been reported in *E. macquarii* (Mackerras, 1961), and its lack of detection may be an indication of low prevalence in this species or parasite preference for *C. longicollis* when both hosts are available. It is also possible that the use of molecular methods to detect hemoparasites may have improved detection of *H. clelandi* infection in *C. longicollis* and *E. macquarii* and contributed to definitive identification (Ujvari et al., 2004). The lifecycle of *H. clelandi* is unknown in Australian chelonians (Jakes et al., 2001).

Intraerythrocytic parasites are commonly encountered in reptiles and are often considered to be an incidental finding (Campbell, 2006). Although infection with hemoparasites may not result in overt clinical disease, it is known to exert marked physiologic costs on hosts, causing anemia, increased circulating immature RBCs, decreased hemoglobin, decreased maximal oxygen consumption, decreased running stamina, smaller testes, less fat storage in preparation for winter dormancy, poor body condition, smaller home ranges, and social submissiveness (Schall, 1982, 1983; Schall and Dearing, 1987; Oppliger et al., 1996; Buoma et al., 2007). Parasitemia in female reptiles has been correlated with poor reproductive output (Schall, 1983; Madsen et al., 2005). Infection with hemoparasites is not always associated with poor health in free-ranging reptiles, especially when hosts are infected with low numbers of parasites (Caudell et al., 2002; Brown et al., 2006). Infection with *H. clelandi* in *C. oblonga* and *C. longicollis* did not appear to influence most blood values, morphology, or reproductive success in these species. The statistically significant decrease in TWBC in *C. oblonga* associated with increased parasite numbers was not considered biologically significant, given that the TWBC was still within reported ranges and turtles did not display signs of clinical disease. The observed eosinophilia in parasitized *C. longicollis* may be associated with hemoparasitism and stimulation of the immune system; however, some species of turtles may have up to 20% circulating eosinophils in normal animals (Campbell and Ellis, 2007). Despite the apparent lack of pathophysiologic effects of hemoparasitism in the chelonians in this study, infection with *H. clelandi* must impose an energetic cost, as parasites are exploiting nutrients that could otherwise be utilized by the host (Hanssen et al., 2003; Oppliger et al., 1996). It is possible that the lack of energetic cost may be due to abundant food resources available to chelonians in this study, facilitating their ability to compensate for the energy or nutrients appropriated by *H. clelandi*. Food availability at capture sites was not assessed as part of this investigation.

Serum biochemistry values for all three species examined were similar to those

previously reported in chelonians with the exception of Ca^{2+} (Anderson et al., 1997; Diethelm and Stein, 2006; Hidalgo-Vila et al., 2007; Innis et al., 2007; Chung et al., 2009; Rangel-Mendoza et al., 2009; Rossini et al., 2010; Chansue et al., 2011). Calcium was notably higher in the chelonians in this study and is likely to reflect egg production and development (Anderson et al., 1997; Harr et al., 2001). The most notable difference in serum biochemistry analyses between species was CK, which was significantly higher in *C. oblonga* than in *C. longicollis* and *E. macquarii.* This may reflect differences in trapping method and transportation. Results of CK analysis for *C. oblonga* were also significantly higher than those reported in other chelonians (Diethelm and Stein, 2006). It is possible that *C. oblonga* may have struggled more in the funnel traps or during transportation, resulting in more muscle damage. Other observed differences in biochemistry values are likely to represent species-specific values.

We attempted to correlate blood values with female morphometric data. Although some analytes were statistically correlated to turtle size, these could not be attributed to biological traits and therefore blood parameters are unlikely to be useful predictors of body size and weight in these species examined.

This is the first baseline study of hematologic and serum biochemical values in wild-caught, gravid *C. oblonga*, *C. longicollis*, and *E. macquarii*. The differences in blood values between species highlight the need for species-specific values when assessing the health of reptiles. Infection with the hemoparasite *H. clelandi* had little physiologic consequences on its chelonian hosts. Blood values were poor predictors of morphologic data. These data provide an insight into the effects of morphology and hemoparasitism on blood parameters of Australian chelonians and the relationship between parasitism and reproductive performance in these species.

ACKNOWLEDGMENTS

We thank the ABAXIS Company for generously donating the biochemistry rotors for this study.

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Submitted 2 August 2011.
Accepted 20 October 2011.

Cheers! Team turtle celebrating a successful night on the beach
Photo taken by passing tourist