MONASH University

THE ROLE OF INCUBATION MOISTURE AND METABOLISM IN SEA TURTLE HATCHLING LOCOMOTOR PERFORMANCE

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The role of incubation moisture and metabolism in sea turtle hatchling locomotor performance

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ABSTRACT

Incubation conditions play a critical role in determining offspring traits in many species and particularly in oviparous reptiles. Research has focused on the role of temperature during incubation, which in sea turtles has been shown to influence developmental rates, morphology, locomotor performance and sex determination. Less attention has been given to other environmental variables such as moisture, despite moisture also varying spatially and temporally throughout incubation. Moisture concentrations during incubation have been shown to influence hatching success, morphology and primary sex ratios but no studies have explored how moisture influences sea turtle hatchling dispersal ability. Thus, I investigated the effect of moisture concentrations during incubation on sea turtle hatchling dispersal ability and considered the ecological ramifications for sea turtle populations.

The ability of sea turtle hatchlings to successfully survive dispersal is determined by numerous factors including their locomotor performance which determines their ability to escape predators and wave zones, their metabolic rates that influence activity levels and yolk consumption, and their thermal tolerance which will become increasingly important as sand and ocean temperatures rise. To measure the response of hatchling dispersal ability to moisture concentrations during incubation, I began by incubating green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*) and flatback sea turtle (*Natator depressus*) eggs at three moisture concentrations before testing hatchling locomotor performance and oxygen consumption during the frenzy and post-frenzy. Hatchlings incubated in dry conditions were slower crawlers than hatchlings incubated in wet conditions, but moisture concentrations did not influence swimming performance. The response of metabolic rates to moisture was inconsistent but when we did observe an effect, dry conditions produced hatchlings with elevated metabolic rates. Thus, reductions in moisture concentrations in sea turtle nests may have negative consequences for the ability of hatchlings to successfully reach the ocean but will not impact the dispersal ability of hatchlings once they enter the water. These hatchlings may also be capable of higher aerobic effort but may be at greater risk of starvation during dispersal.

To determine the response of hatchling thermal tolerance to moisture concentrations during incubation, I incubated green sea turtle eggs in a hatchery on a natural beach at low and high moisture. As hatchlings emerged, I measured their hydration levels and critical thermal maximum, neither of which were influenced by moisture concentrations during incubation.

However, hatchlings that had longer incubation durations also had lower thermal tolerance. Using incubation duration as a proxy for incubation temperature, it appears that hatchlings acclimate to nest temperatures and therefore, hatchlings from warmer nests have higher thermal tolerance. Thus, hatchlings may be able to acclimatise to warming nesting beaches but the extent to which they can adapt remains unknown.

Lastly, I measured the ontogeny of hatchling metabolic rates and swimming performance and examined species differences to understand the long-term implications of altered incubation conditions on sea turtle populations. Variation in both metabolic rates and swimming performance among sea turtle species largely reflected differences in life history. For example, green sea turtle hatchlings had the highest routine and maximal metabolic rates and they also maintained elevated swimming activity levels up to 24 weeks post-frenzy, suggesting that they may undertake extended dispersals from nesting beaches. Comparatively, leatherback hatchlings exhibited low metabolic rates during routine swimming, but high resting metabolic rates, likely reflecting their slow, pelagic foraging behaviours. Thus, changes in dispersal ability will impact species differently depending on their life history.

In summary, dry incubation conditions produce hatchlings that are slower crawlers and self-righters, but may also have elevated metabolic rates. Thus, hatchlings incubated in dry conditions may be at greater risk of predation as they crawl to the ocean, and they may be at greater risk of starvation during dispersal, but they are likely to have greater thermal tolerance than hatchlings incubated in wet conditions. The ability to tolerate elevated temperatures may outweigh reduced locomotor performance, particularly in the light of climate change. The overall effect of these responses on population dynamics will depend differences in life histories among species, nesting beach characteristics and the ability of nesting females to adjust nest site selection and the timing of nesting.

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Thesis declaration

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

This thesis includes two published manuscripts and three unpublished manuscripts. The core theme of the thesis is *the role of incubation moisture and metabolism in sea turtle hatchling locomotor performance.* The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Richard Reina and Jeanette Wyneken.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. Therefore, I present the following chapters as prepared for submission to peer-reviewed journals.

In the case of chapters 2-6 my contribution to the work involved the following:

Thesis chapter	Publication title	Status	Student contribution	Co-author contribution(s)	Co-author(s) Monash student?
2	A review of incubation conditions and their effects on hatchling phenotypes in the Reptilia	In review at *BioScience*	85% Literature search and writing of the manuscript	Richard Reina[c] 15%	No
3	Sea turtle hatchling locomotor performance: Incubation moisture effects, ontogeny and species-specific patterns	In press at the *Journal of Comparative Physiology B*	90% Experimental design, data collection, data analysis and writing of the manuscript	Richard Reina[ac] 10%	No
4	The role of incubation environment in determining sea turtle thermal tolerance	In review at *Physiological and Biochemical Zoology*	85% Experimental design, data collection, data analysis and writing of the manuscript	1. Bill Matthews[bc] 5% 2. Richard Reina[ac] 10%	Yes *BM
5	Ontogeny and ecological significance of metabolic rates in sea turtle hatchlings	Submitted to *Functional Ecology*	75% Experimental design, data collection, data analysis and writing of the manuscript	1. T Todd Jones[abc] 10% 2. Brittany Imlach[abc] 5% 3. Richard Reina[ac] 10%	No
6	The ontogeny of sea turtle hatchling swimming performance	In press at the *Biological Journal of the Linnean Society*	90% Experimental design, data collection, data analysis and writing of the manuscript	Richard Reina[ac] 10%	No

[a] Helped develop experimental design

[b] Helped with data collection

[c] Proofread and contributed to the manuscript

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:** 30/04/2020

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

Main Supervisor signature: **Date:** 30/04/2020

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First and foremost, I would like to thank my supervisor, Richard Reina. Frank talked you up in Costa Rica and you lived up to the hype. Your insights and advice on my experimental design, analysis and writing have made every aspect of this thesis better and I am a better researcher and writer for it. I'm sure that my project caused you a fair bit of stress along the way, but you were always there, and I really appreciated your consistent support. You have taught me not only how to be a better scientist, but you have also set a great example on how to maintain a healthy work-life balance and I hope I can follow your lead. Thank-you for everything and I look forward to many more symposium beers in the future.

Jeanette, we haven't spent much time in the same country but your impact from across the seas has still been great. I've truly appreciated your advice, guidance and support throughout my degree. It has been fantastic getting to know you at the symposiums in Kobe and Charleston and I can see why you are in such high demand from everyone in the sea turtle world. I am particularly grateful to you for opening up your lab to me for my Fulbright application. Hopefully I'll make it to FAU in the future!

To Fran, thanks for all of the trips, nights on the beach, free veterinary advice and hours in the CT room. I know that my project took up a lot of your time and that things didn't always go to plan, but I really appreciate all of the hours that you spent helping me out. Though I have one suggestion for you, it may be time to cut down on the salt intake!

I have been fortunate enough to travel around Australia and the world completing this project. I have been even more fortunate to work with some incredible people each step of the way.

Thank-you to everyone at the Heron Island research station for your help collecting green sea turtle eggs and releasing hatchlings.

Thank-you to Col Limpus and John Sergeev for organising my trip to Curtis Island to collect flatback eggs. To Erwin and Fiona Hoffman, Maree McLaren and Brendan Lumley, I had an absolute blast working with you and I appreciate all of your help, especially on that last night where we finally got some turtles. Thanks Erwin for putting me in contact with the Gladstone Volunteer Marine Rescue, I'm sorry that you and Brendan couldn't join Maree, Fiona and I when we released the hatchlings.

I never expected that I would get the opportunity to visit the Tiwi Islands, let alone get up close to the turtles (and the crocodiles) there. Thank-you to the Tiwi Land Council for giving me permission to visit your ancestral lands and to Kate Hadden for all of your help. I would especially like to say thank-you to James for looking after us while collecting eggs. You are an absolute legend.

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Gladstone with me to release hatchlings. I had a great time on that trip, and I hope you did too. Really, just thank-you for being you. I love you.

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Chapter 1. General Introduction

A female flatback sea turtle returning to sea after laying on Curtis Island.

Photo taken by Christopher Gatto.

1.1 BRIEF OVERVIEW

Animal species utilise a spectrum of reproductive strategies, ranging from eggs developing directly in the external environment to the production of live young that are continuously supplied with nutrients as they develop entirely within the mother (Lodé, 2012). Generally, this suite of reproductive strategies is divided into two main groups: oviparity (i.e. egg laying where the majority of development is in the external environment) and viviparity (i.e. live young bearing species where embryonic development occurs internally) (Blackburn, 1999). Viviparity, while providing the most control over an embryo's developmental environment and allowing mothers to protect their offspring from sources of mortality (Webb *et al.*, 2006), comes with inherent disadvantages, including increased energy demands and inhibition of the mother's locomotor ability (Lin *et al.*, 2008; Schultz *et al.*, 2008). Conversely, oviparity is less energetically costly and allows for increased fecundity, but exposes developing embryos to the unpredictability of the external environment (Blackburn, 1999).

While many oviparous species utilise behavioural tactics, such as brooding in snakes (Lourdais *et al.*, 2007; Brashears & DeNardo, 2013), to minimise environmental fluctuations during embryonic development, the majority of reptiles provide no parental care (Balshine, 2012). Therefore, reptilian embryos experience considerable variation in their external environment with significant implications for their development. Most research has investigated the effects of thermal variation during incubation on embryonic development and the traits of the resultant offspring (Allsteadt & Lang, 1995; Alberts *et al.*, 1997; Booth, 1999; Andrews, 2008; Mickelson & Downie, 2010; Booth, 2017). In the oviparous reptilians, differences in thermal conditions during incubation influence embryonic survival and growth rates and in the resultant offspring can influence size, morphology, locomotor ability, metabolic rates and sexual differentiation (Ewert *et al.*, 1994; Booth, 2017; Noble *et al.*, 2017).

In particular, the effect of incubation temperature on sexual differentiation has received extensive attention and is the subject of numerous reviews (Lang & Andrews, 1994; Wibbels, 2003; Gamble, 2010; Rhen & Schroeder, 2010; Merchant-Larios & Diaz-Hernandez, 2013). Sex determination patterns vary among taxa (Viets *et al.*, 1994; Shine, 2003; Mitchell *et al.*, 2006) and pivotal temperatures vary among both species (Wibbels, 2003) and populations (Ewert *et al.*, 2005; Refsnider *et al.*, 2014). The influence of incubation temperatures on sex ratios has received particular attention because warming nest temperatures have been

projected to result in biased primary sex ratios, eventually altering adult sex ratios and reducing population viability (Fuentes *et al.*, 2010; Kallimanis, 2010; Mitchell & Janzen, 2010; Fuentes *et al.*, 2011).

However, recent studies have suggested that warmer incubation conditions are more likely to reduce population viability by increasing embryonic mortality (Hawkes *et al.*, 2007; Santidrián Tomillo *et al.*, 2012; Pike, 2014; Hays *et al.*, 2017). Hatching success is maximised at intermediate temperatures among the *Reptilia* with reduced hatching success at cooler and warmer temperatures (Piña *et al.*, 2003; Booth *et al.*, 2004; Nelson *et al.*, 2004). Development rates are faster at higher temperatures resulting in shorter incubation durations and smaller hatchlings (Hutton, 1987; Van Damme *et al.*, 1992; Booth & Astill, 2001a; Burgess *et al.*, 2006). Additionally, warmer incubation temperatures generally produce smaller hatchlings in most taxa other than crocodilians (Webb & Cooper-Preston, 1989; Booth & Evans, 2011; Monasterio *et al.*, 2013) and hatchling locomotor performance is maximised at intermediate incubation temperatures (Elphick & Shine, 1998; Noble *et al.*, 2017; Booth, 2018). Incubation temperatures that produce smaller, weaker hatchlings will result in elevated mortality rates because smaller, slower offspring are more vulnerable to predators (Ferguson *et al.*, 1982; Civantos *et al.*, 1999; Gyuris, 2000; Steer *et al.*, 2003; Cavallo *et al.*, 2015). Reptile locomotor performance, behaviour and metabolic rates can be influenced by ambient temperatures (Southwood *et al.*, 2003a; Southwood *et al.*, 2003b; Southwood *et al.*, 2006; Kearney *et al.*, 2009; Rodgers *et al.*, 2015), but generally, changes in ambient conditions do not mitigate the effects of altered incubation conditions (Booth & Evans, 2011; Cavallo *et al.*, 2015).

This variation in hatchling traits as a result of thermal variation during incubation occurs spatially because of geographic differences among populations and because of differences in nest site selection within populations (Stokes *et al.*, 2006; Zbinden *et al.*, 2007). It can also occur temporally, among nesting seasons because of stochastic yearly variation or within nesting seasons, particularly in species that nest over multiple months (Warner *et al.*, 2010). Research has particularly focussed on investigating how future climate change is likely to alter offspring traits and therefore, population viability (Hawkes *et al.*, 2009; Fuentes *et al.*, 2010; Witt *et al.*, 2010; Laloe & Hays, 2017). Warmer nest temperatures have been predicted to increase embryonic mortality, lead to smaller, weaker offspring and create biased primary sex ratios in reptile species with environmental sex determination, leading to reduced

hatchling recruitment into adult populations and eventual population extinction (Hawkes *et al.*, 2007; Telemeco *et al.*, 2009; Wapstra *et al.*, 2009; Cavallo *et al.*, 2015; Santidrián Tomillo *et al.*, 2015; Laloë *et al.*, 2017).

The current focus on temperature has led to a limited understanding of how environmental variation impacts embryonic development and offspring traits. Less attention has been paid to the roles that other environmental factors, such as moisture, play in determining hatchling traits and consequently, their impact on population dynamics and viability. Aside from temperature, moisture has received the most attention from researchers. Reptile species incubated in wet conditions are generally faster swimmers and crawlers than those incubated in dry conditions (Miller *et al.*, 1987; Miller, 1993; Finkler, 1999; Brown & Shine, 2006). Although, tropical species are more responsive to moisture levels during incubation than species from more arid zones (Flatt *et al.*, 2001; Warner & Andrews, 2002). Turtle (Reece *et al.*, 2002; Bodensteiner *et al.*, 2015), snake (Brown & Shine, 2006; Brown & Shine, 2018) and lizard hatchlings (Du & Shine, 2008; Xiao-long *et al.*, 2012) tend to be larger and longer when incubated in wetter incubation conditions, although studies on crocodiles are limited. Although the mechanisms remain unknown, elevated moisture concentrations during incubation resulted in embryos converting more yolk mass into hatchling mass (Christian *et al.*, 1991; Hewavisenthi *et al.*, 2001) and thus, larger and longer hatchlings.

Studies have only recently begun investigating the effects of moisture during incubation on sex determination. Although recent research has found a relationship between moisture concentrations and primary sex ratios, it remains unclear whether moisture has a direct effect on sex determination or whether it has an indirect effect by altering incubation temperatures or restricting oxygen availability (Lolavar & Wyneken, 2015; Cedillo-Leal *et al.*, 2017; Lolavar & Wyneken, 2017). However, numerous studies have found no relationship between moisture concentrations during incubation and primary sex ratios (Packard, 1991; Bobyn & Brooks, 1994; Hewavisenthi & Parmenter, 2000), suggesting that the effect of moisture on sex determination is likely to be indirect. Overall, temperature appears to have the greatest effect on hatchling traits and development, although moisture can also influence hatchling traits directly and can interact with temperature to alter hatchling responses to incubation temperatures (Ackerman *et al.*, 1997). However, unlike mean temperatures that are expected to increase globally, changes in most other environmental factors are predicted to vary regionally (Pachauri *et al.*, 2014). Therefore, embryonic and hatchling responses to these

environmental factors are also likely to vary regionally. Expanding our understanding of how environmental factors other than temperature influence hatchlings traits will improve our knowledge of how and why hatchlings from different nests and populations differ in quality.

Variation in hatchling quality, because of incubation conditions, has important consequences for hatchling survival. Four critical traits that determine reptile hatchling survival are size, locomotor performance, thermal tolerance and hatchling energetics. Hatchling size not only influences locomotor performance (Miles *et al.*, 1995; Burgess *et al.*, 2006; Booth & Evans, 2011) but can also limit the ability of predators to consume hatchlings as predators become gape limited (Webb & Shine, 1993; Persson *et al.*, 1996; Gyuris, 2000). Hatchlings that are larger are likely to be faster locomotors and are less likely to be predated (Miles, 2004), but generally emerge with smaller yolk reserves than smaller hatchlings (Allsteadt & Lang, 1995; Gyuris, 2000; Booth & Astill, 2001b; Radder *et al.*, 2004). Sea turtles have been the focus of many studies on the effects of incubation conditions on hatchling quality and dispersal ability because sea turtle hatchlings experience intense predation rates during dispersal from their nesting beaches. Predation rates are particularly high during the hatchling's initial crawl from the nest to the ocean and in nearshore, neritic waters (Gyuris, 1994; Santidrián Tomillo *et al.*, 2010). Hatchlings that are slower swimmers, irrespective of size, and spend more time in neritic waters are more likely to be predated than hatchings that quickly escape to deeper pelagic waters that are less predator-dense (Gyuris, 1994). Thus, hatchling size and locomotor performance strongly influence the opportunity for predators to consume hatchlings and the duration of time that hatchlings are exposed to high concentrations of these predators.

However, variation in hatchling size and locomotor performance as well as incubation conditions also have important consequences for hatchling energetics. Sea turtle hatchlings survive solely on yolk reserves for approximately a week post-emergence (Jones *et al.*, 2007). Larger hatchlings generally emerge with smaller yolk reserves (Booth & Astill, 2001b) and must begin feeding sooner than smaller hatchlings with larger residual yolks (Kraemer & Bennett, 1981). Hatchlings that exhibit elevated metabolic rates are also likely to consume yolk reserves at a faster rate, placing them at greater risk of starvation than hatchlings with lower metabolic rates (Kraemer & Bennett, 1981; Jones *et al.*, 2007). However, higher metabolic rates allow hatchlings to maintain elevated activity levels (Booth, 2009) and potentially grow faster (Reid *et al.*, 2009; Burton *et al.*, 2011), reducing mortality rates compared to hatchlings with lower metabolic rates. Thus, hatchling energetics play an

important role in determining hatchling survival; indirectly by influencing growth and locomotor performance, as well as directly by determining rates of yolk utilisation in dispersing hatchlings.

Dispersal is a critical time for sea turtles because of the intense predation rates experienced by hatchlings during this period (Gyuris, 1994). Hatchling size, locomotor performance, thermal tolerance and metabolic rates all contribute to the ability of hatchlings to successfully disperse from nesting beaches and enter pelagic waters. However, these traits are likely influenced by incubation conditions and thus, exhibit considerable variation within and among nesting seasons and populations. While recent studies have begun to investigate the effects of temperature on hatchling dispersal ability (Burgess *et al.*, 2006; Booth, 2017; Booth, 2018), few studies consider the effects of other environmental factors, such as moisture. If particular moisture concentrations during incubation produce hatchlings that are poorer dispersers, then these hatchlings may be at greater risk of predation and are less likely to be recruited into adult populations (Cavallo *et al.*, 2015). Thus, determining how moisture concentrations influence hatchling dispersal ability, combined with our knowledge of the effects of temperature, provides us with a greater understanding of how spatial and temporal variation in incubation conditions, as well as climate change, are likely to alter sea turtle population dynamics and viability.

1.2 STUDY SPECIES

Sea turtle nesting seasons extend over many months, with individual females laying multiple clutches of eggs per season. While females do not nest every year, their reproductive lifespans can last for decades (Miller, 2017). This unique combination of high reproductive output per nesting season and long reproductive lifespan means that any two clutches of eggs laid in either the same or different nesting seasons may experience drastically different incubation conditions. These differences can be the result of climatic variation among years, seasonal change within nesting seasons and spatial differences among and within nesting beaches caused by variation in shade, rainfall, proximity to the ocean and plant density (Ackerman *et al.*, 1997; Stokes *et al.*, 2006; Zbinden *et al.*, 2007; Warner *et al.*, 2010). Additionally, sea turtles provide no parental care and dispersing hatchlings can experience high mortality rates, thus variation in hatchling quality directly impacts hatchling survival (Gyuris, 1994; Janzen *et al.*, 2000; Pilcher *et al.*, 2000; Salmon *et al.*, 2009; Duran & Dunbar, 2015). Considering that sea turtle hatchling traits respond strongly to temperature variation

during incubation and that these traits directly influence hatchling survival, sea turtle population dynamics and viability are highly dependent on environmental conditions on nesting beaches (Saba *et al.*, 2012; Santidrián Tomillo *et al.*, 2012).

For this thesis, I collected and incubated eggs from green sea turtles (*Chelonia mydas*), flatback sea turtles (*Natator depressus*) and olive ridley sea turtles (*Lepidochelys olivacea*). Green sea turtle eggs were collected from Heron Island, Queensland (chapters 3 and 6) and Terengganu, Malaysia (chapters 4 and 5). The females sampled on Heron Island were part of the Southern Great Barrier Reef breeding unit and are likely to have migrated from feeding grounds ranging along the East Australian coast from Papua New Guinea to New South Wales, but may have migrated from as far east as New Caledonia and Fiji. Nesting for this rookery runs from October to late March with hatchlings emerging from December to May. Females lay 3-7 clutches of approximately 115 eggs every 3-7 years. Eggs are approximately 47g with hatchling mass being around 25g (Limpus & Fien, 2009). The females sampled in Terengganu were part of the Peninsular Malaysia Management Unit, though these turtles do not differ genetically from females that nest in the Philippines (Moritz *et al.*, 2002). Females from this population generally forage within the waters of South-East Asia including Malaysia, Indonesia and the Philippines (Liew *et al.*, 1995). Sea turtles in Malaysia and South-East Asia face numerous threats, including fisheries capture, harvesting for meat and the systemic collection of eggs (Shanker & Pilcher, 2003).

Olive ridley turtle eggs were collected from the Tiwi Islands, Northern Territory. Less studied than green sea turtles, olive ridley nesting appears to occur year round in northern Australia and females are likely to forage within the Australian continental shelf (Whiting *et al.*, 2005). Nesting details for Australian olive ridley turtles are scarce, but northern Australian populations lay approximately 105 eggs. Overseas populations generally lay 1-2 clutches every 1-3 years, with olive ridleys laying fewer clutches per nesting season, but returning to nest more frequently than other sea turtle species. They also lay the smallest eggs at approximately 30g and produce the smallest hatchlings at approximately 15g (Limpus & Fien, 2009).

Flatback sea turtle eggs were collected from Curtis Island, Queensland and are part of the Eastern Australian management unit. Foraging grounds for this population are almost exclusively within the Great Barrier Reef World Heritage Area but can extend to the Torres

Strait. Additionally, flatback sea turtles exhibit a completely neritic life history, with migration distances for flatbacks being shorter than other sea turtle species (Bolten, 2003). Nesting in this population occurs from October to January with hatchlings emerging from December to March. Females lay 2-4 clutches of approximately 50 eggs every 2-4 years. Clutch size in flatbacks is considerably less than those of olive ridleys or green turtles, but egg size at approximately 78g is significantly larger as is hatchling size at approximately 44g (Limpus & Fien, 2009).

These three species were selected for the differences in their life histories that are most likely to influence their response to moisture during incubation such as egg size and for differences in their hatchling dispersal behaviours. Furthermore, these species were selected because they naturally experience variation in moisture levels on nesting beaches, are likely to experience changes to these moisture regimes under climate change and they have a high likelihood of successful artificial incubation of their eggs.

1.3 STUDY AIMS

The broad aim of my study was to investigate how moisture levels during incubation influence hatchling locomotor performance in sea turtles, in order to broaden our understanding of how environmental conditions determine hatchling recruitment and influence population dynamics. To achieve this, I designed a series of experiments (chapters 3-6) that addressed specific questions on the consequences of moisture variation during incubation for sea turtle hatchlings and populations (Figure 1.1). Sea turtle hatchling traits have been shown to respond strongly to variation in incubation temperatures, while closely related freshwater turtles have been the main focus of the few studies investigating the effects of moisture during incubation (Packard *et al.*, 1987; Packard *et al.*, 1988; Packard *et al.*, 1989; Packard *et al.*, 1991). Sea turtle species nest on coastal beaches where moisture levels can vary significantly and are expected to change considerably under climate change (Pachauri *et al.*, 2014). Thus, sea turtles were an ideal model species for this study. Additionally, producing high quality offspring is paramount for sea turtles because hatchlings experience high mortality rates during dispersal from the nest and receive no parental care (Gyuris, 1994; Janzen *et al.*, 2000; Pilcher *et al.*, 2000; Salmon *et al.*, 2009). Therefore, changes to hatchling locomotor performance can have significant and direct effects on hatchling recruitment, population dynamics and population viability. Finally, I investigated ontogenetic changes in hatchling locomotor performance in order to gain a broad

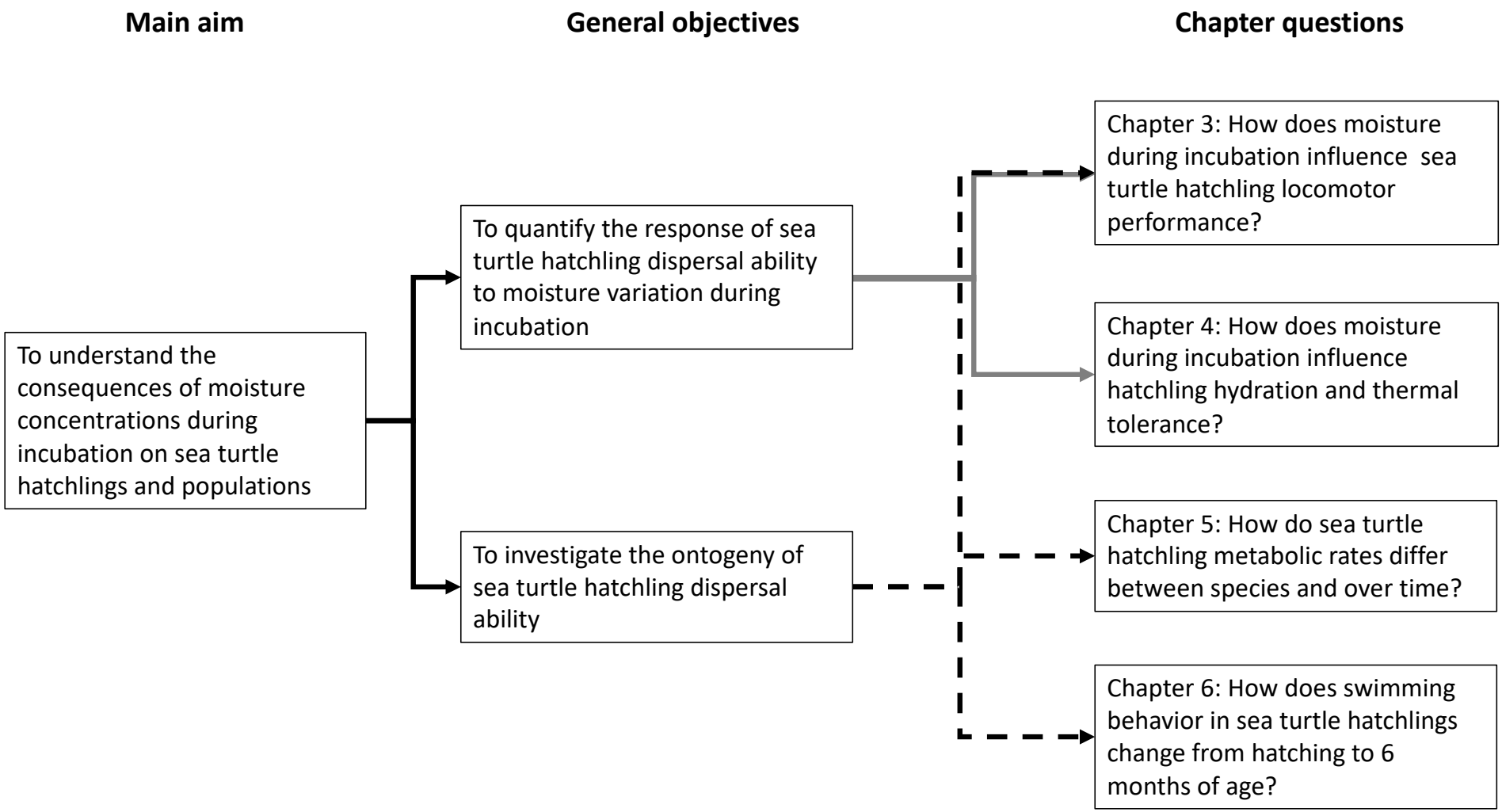

Figure 1.1: Main research aims and general thesis structure.

understanding of how changes in moisture levels on nesting beaches may influence sea turtle populations, rather than focusing solely on the initial dispersal of hatchlings from nesting beaches. This thesis focuses on three lesser-studied traits that determine dispersal ability in sea turtle hatchlings. It investigates the effects of moisture on locomotor performance and thermal tolerance in order to understand how temporal and spatial variation in moisture influences dispersal ability. It also investigates ontogenetic changes in locomotor performance and metabolic rates to provide further insight into the long-term consequences of altered incubation conditions and variation in dispersal ability on hatching survival. Finally, this thesis reports on the effects of moisture concentrations during incubation on morphology, hatching success and incubation duration.

1.4 THESIS STRUCTURE

This thesis consists of a general introduction, five chapters and a general discussion. It consists of research that came from active collaboration and team-based research. Therefore, I present the following chapters as prepared for submission to peer-reviewed journals. This general introduction is brief because **chapter two** is a literature review that details our current understanding of how multiple environmental factors influence a variety of hatchling traits and embryonic development in the major reptilian orders. Additionally, I discuss how these environmental factors interact within nests and the consequences of these interactions for hatchling traits. Finally, I explore the impact that current and future climatic variation could have on hatchling recruitment and adult populations and finish by recommending future research directions.

In **chapter three,** I empirically quantify the response of various hatchling traits to ecologically relevant moisture levels during incubation. By taking a comparative approach, I am also able to consider inter-species differences in these traits and I explore the potential consequences for population dynamics and viability. Chapter three focuses on terrestrial and aquatic locomotor performance and also investigates the effect of moisture on hatching success, incubation duration and hatchling morphology. In this chapter, I test hatchlings at hatching and at 4 weeks of age allowing me to investigate the long-term effects of moisture concentrations on hatchling locomotor performance and morphology.

In **chapter four**, I explore the effects of moisture during incubation on hatchling hydration and thermal tolerance. I also comment on the potential consequences for population dynamics

and viability. This study was conducted in Terengganu, Malaysia using green sea turtle eggs incubated in a shaded hatchery on a natural beach.

Chapters five and six investigate the ontogeny of sea turtle hatchling metabolic rates and locomotor performance. Thus, they investigate how hatchling dispersal ability changes over time, providing insight into how changes in dispersal ability, as a result of incubation conditions, may influence hatchling recruitment, population dynamics and population viability.

In chapter five, I combined my measurements of metabolic rates with unpublished data on metabolic rates in loggerhead, leatherback and green hatchlings. By combining these data with the metabolic rate data I collected on green, olive ridley and flatback turtles, I was able to compare the ontogeny of metabolic rates in multiple sea turtle species. This allowed me to further evaluate differences among species and how alterations to moisture levels during incubation may affect species' metabolic rates and hatchling recruitment differently.

Chapter six arose from chapter four that focussed on the effect of moisture on locomotor performance. It concentrates on the ontogeny of swimming performance in green sea turtle hatchlings from hatching to 24 weeks of age. Additionally, this chapter investigates which swimming behaviours have the strongest influence on overall swimming ability, providing further insight into how altered swimming behaviours, as a result of different moisture levels during incubation, could influence overall swimming ability and hatchling recruitment.

Finally, **chapter seven** synthesises all of the chapters into a single general discussion on the overall findings of this thesis. It considers the implications of temporal and spatial variation in hatchling traits as a result of altered incubation conditions for our understanding of population dynamics, for conservation and for population management. Lastly, I consider study limitations and avenues for future research.

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Chapter 2. A review of incubation conditions and their effects on hatchling phenotypes in the Reptilia

A green sea turtle embryo is candled in the lab at Monash University. Photo taken by Cristina Chang.

In review at *BioScience*

2.1 ABSTRACT

Developing embryos of oviparous reptiles show substantial plasticity in their responses to environmental conditions during incubation. Variable conditions can alter sex ratios, morphology, locomotor performance and hatching success. While recent research and reviews have focused on temperature during incubation, emerging evidence suggests other environmental variables are important in determining hatchling phenotypes. Understanding how the external environment influences development is important for species management and requires identifying how environmental variables exert their effects individually, and how they interact to affect developing embryos. To address this knowledge gap, we review the literature on phenotypic responses in oviparous reptile hatchlings to temperature, moisture, oxygen concentration and salinity. We examine how these variables influence one another and consider how changes in each variable alters incubation conditions and thus, hatchling phenotypes. We explore how incubation conditions drive variation in hatchling phenotypes and influence adult populations. Finally, we highlight knowledge gaps and suggest future research directions.

2.2 INTRODUCTION

Animals can increase their reproductive fitness by optimising offspring quantity and quality (Olsson & Shine, 1997; Einum & Fleming, 2000; Charnov & Ernest, 2006). Species are typically described as ranging from r-selected, where offspring number is maximised, to K-selected, where offspring quality is maximised but few individuals are produced (Pianka, 1970). Different taxa employ a range of strategies to vary their energetic investment in mate selection (Jennions & Petrie, 1997), allocation of resources to reproduction (Stearns, 1989; Charnov & Ernest, 2006), parental care (Webb *et al.*, 1999) and the developmental environment of their offspring (Hays *et al.*, 1995; Blackburn, 1999; Doody *et al.*, 2006). However, resources are finite, limiting the ability to allocate maximum resources to all aspects of reproduction. Thus, each reproductive strategy represents a trade-off in resources between the selected strategy and other potentially beneficial strategies (Ebert, 1993; Van Buskirk & Crowder, 1994; Wallace *et al.*, 2007).

A species' ability to adaptively select between the quantity and quality of their offspring is limited environmentally and physiologically, including by its reproductive mode (Wiens, 1984; Reznick *et al.*, 1990; Roff, 1993; Stearns, 2000)**.** Reproductive life-history modes can broadly be described as ranging from oviparity with little or no parental care to viviparity

752 with parental care, and a variety of intermediate forms (Lodé, 2012). Modes generally differ
753 in their degree of parental investment in individual offspring, with a trade-off between
754 offspring number and probable survival rate (Blackburn, 1999). Non-brooding oviparous
755 females minimise the time spent burdened by eggs, both physically and physiologically,
756 enabling females to increase the number and size of clutches laid (Blackburn, 1999).
757 However, this mode exposes eggs to variations in the incubation environment, including
758 unfavourable conditions that may negatively affect embryonic development (Rana, 1990;
759 Angilletta *et al.*, 2000).
760 While many oviparous species have evolved adaptations (e.g. ovoviviparity or post-
761 ovipositional brooding) to reduce environmental variation for developing eggs and embryos,
762 most reptile species do not provide any parental care during or after incubation (Balshine,
763 2012). For oviparous reptiles with little or no parental care, the timing of oviposition and
764 location of clutches can have implications for incubation conditions and therefore, the quality
765 and quantity of resultant offspring (Kolbe & Janzen, 2002; Kamel & Mrosovsky, 2004; Li *et*
766 *al.*, 2018).
767 Research into how different incubation environments influence reptile hatchling phenotypes
768 has been extensive (e.g. Gutzke *et al.*, 1987; Hutton, 1987; Ashmore & Janzen, 2003; Bell *et*
769 *al.*, 2013; Booth, 2017) and the significance of variation in incubation environments is clear
770 (Nelson *et al.*, 2004a; Hamann *et al.*, 2010; Rees *et al.*, 2016). However, the majority of this
771 research has focussed on the phenomenon known as temperature-dependent sex
772 determination (TSD), which occurs in all reptile taxa except snakes (Shine, 2003).
773 Temperature has been shown to influence population viability by affecting the primary sex
774 ratios of developing embryos (Burger & Zappalorti, 1988; Mrosovsky, 1994; Hanson *et al.*,
775 1998; Hawkes *et al.*, 2007; Fuentes *et al.*, 2010; Kallimanis, 2010; Mitchell & Janzen, 2010)
776 as well as hatchling traits, such as locomotor performance and morphology (Booth & Evans,
777 2011; Wood *et al.*, 2014; Cavallo *et al.*, 2015). In contrast, much less attention has been paid
778 to the impacts of other environmental factors, such as moisture, oxygen concentration and
779 salinity, on hatchling phenotypes. Without this information, it is difficult to predict with any
780 certainty 1) how hatchling phenotypes will respond to changes in complex environmental
781 systems and 2) the potential consequences for adult populations (Díaz-Paniagua & Cuadrado,
782 2003; Brown & Shine, 2006).
783 There is a clear need to investigate how environmental factors influence hatchling
784 phenotypes and how these effects may vary among oviparous reptile taxa (hereafter
785 'reptiles'). The role of temperature in determining sex and sex ratios is reviewed extensively

elsewhere (e.g. Warner & Shine, 2008a; Warner, 2011; Georges & Holleley, 2018) and thus is not a major theme of our review. We instead focus on how moisture, oxygen concentration and salinity influence hatchling phenotypes and developmental success in a wide range of reptile taxa. We discuss how these environmental factors can interact to determine phenotypes and explore the impact that climatic variation may potentially have on hatchling recruitment and population viability. Finally, we recommend future research directions to address under-represented biological topics or taxonomic areas.

2.3 EFFECTS OF INCUBATION CONDITIONS ON HATCHLING PHENOTYPES

Incubation conditions are largely dependent upon the type of environment in which eggs are deposited. Reptiles exhibit substantial variety in egg-laying preferences across taxa: underground nests (Miller *et al.*, 2003); aboveground mounds or nests that flood (Kennett *et al.*, 1993); in stumps, tree hollows, on the ground or in leaf litter (e.g., rough green snakes (*Opheodrys aestivus*) (Plummer, 1990), whistling lizards (*Calotes liolepis)* (Karunarathna *et al.*, 2009)). Each of these preferences has consequences for one or more environmental variables affecting the nest microenvironment. For example, shallow or aboveground nests are likely to experience greater fluctuations in temperature than those laid deep underground (Booth, 2006), with deeper nests typically warmer than the ambient air temperature due to metabolic heating (Seymour & Ackerman, 1980; Sieg *et al.*, 2011). In this section, we review how developing embryos are affected by variations in environmental factors during incubation. Effects of geographically large-scale climatic variation are beyond the scope of this review. Additionally, some reptile species do provide parental care such as brooding or nest guarding (Balshine, 2012). While brooding can reduce fluctuations in the incubation environment of developing embryos (Somma & Fawcett, 1989; Lourdais *et al.*, 2007; Stahlschmidt & DeNardo, 2010) and alter offspring traits (Shine *et al.*, 1997; Aubret *et al.*, 2005; Lourdais *et al.*, 2007), reducing fluctuations in the incubation environment are not always advantageous for offspring (Ashmore & Janzen, 2003; Stahlschmidt & DeNardo, 2008; Stahlschmidt & DeNardo, 2009). We focus on the direct effects of altered incubation conditions on developing embryos and thus, the effect of parental care on incubation conditions, offspring phenotypes and offspring survival is beyond the scope of this review.

2.3.1 Temperature

2.3.1.1 Sex ratio

Temperature has been the most studied environmental factor influencing hatchling phenotypes in reptiles. In particular, research has focused on the effect of temperature on sex determination (i.e. TSD), and there are a number of detailed reviews on reptile sex ratio responses and likely mechanisms (Lang & Andrews, 1994; Wibbels, 2003; Gamble, 2010; Rhen & Schroeder, 2010; Merchant-Larios & Diaz-Hernandez, 2013). Here we provide a brief overview of observed temperature-sex patterns within the Reptilia.

While many studies report the effects of temperature on lizard and turtle species, there is limited knowledge of temperature-related effects on hatchling phenotypes for other reptile taxa (e.g. crocodilians, tuataras).

There are three main patterns in the response of sex to temperature. FMF (female-male-female) is a pattern in which males are observed at intermediate temperatures and females at higher and lower temperatures. FM (female-male) and MF (male-female) patterns only transition between the sexes once, with FM species producing females at lower temperatures and MF species producing females at higher temperatures. FMF is the only pattern that is observed in all three major reptile lineages (i.e. Crocodilia, Testudines and Squamata) and is thought to be the ancestral form of TSD (Viets *et al.*, 1994). Many species including crocodilians (Hutton, 1987) and agamids (Harlow & Taylor, 2000) were initially believed to be FM but were later shown to be FMF (Lang & Andrews, 1994).

Squamates display the greatest diversity in their sex determination patterns, showing both TSD and genetic sex determination (Viets *et al.*, 1993; Pokorna & Kratochvil, 2009; Gamble, 2010; Inamdar & Seshagiri, 2012; Santoyo-Brito *et al.*, 2017), although no snakes (Serpentes) are currently known to exhibit TSD (Shine, 2003). Additionally, some squamates have genetic sex determination that can be overridden by temperature (Holleley *et al.*, 2015).

Testudines generally display MF patterns of TSD (Okada *et al.*, 2010; Burke & Calichio, 2014), though some species show FMF patterns (Ewert *et al.*, 1994) and display genetic sex determination (Ji *et al.*, 2003). Rhynchocephalia, consisting of the only extant tuatara species (*Sphenodon punctuatus*), is exclusively FM (Mitchell *et al.*, 2006; Marcó *et al.*, 2017).

TSD occurs during the temperature sensitive period, which is generally the middle third of incubation in most reptiles (Bull, 1987). Pivotal temperatures—the range at which a clutch produces 50% males and 50% females—have been studied most extensively in sea turtles (MF pattern) (Wibbels, 2003; Godfrey & Mrosovsky, 2006; Dobbs *et al.*, 2010; King *et al.*, 2013). Most species have pivotal temperatures between 29°C and 30°C (Table 2.1), which

Table 2.1: The temperature-dependent sex determination (TSD) patterns and pivotal temperatures of various oviparous reptile orders. For species without a specific pivotal temperature, the best approximation (range of temperatures) are given.

Order	Family	Species	TSD Pattern	Pivotal temperature/s (°C)	Reference
Squamata	Eublepharidae	*Eublepharis macularius*	FMF	31 & 33?	Viets *et al.* (1993); Gamble (2010)
		Hemitheconyx caudicinctus	FM	30.5	Viets *et al.* (1994)
	Agamidae	*Calotes versicolor*	FMFM?	23.5, 25.5, 31.5, 34	Inamdar and Seshagiri (2012)
		Physignathus lesueurii	FMF	25 & 28	Doody *et al.* (2006)
	Iguanidae	*Crotaphytus collaris*	MFM?	~28 & ~33.5	Santoyo-Brito *et al.* (2017)
Testudines	Chelonidae	*Eretmochelys imbricata*	MF	29.2	Dobbs *et al.* (2010)
		Eretmochelys imbricata	MF	29.2-29.6	Wibbels (2003)
		Chelonia mydas	MF	29.2-29.3	Godfrey and Mrosovsky (2006)
		Chelonia mydas	MF	28.8-30.3	Wibbels (2003)
		Chelonia mydas	MF	~29	King *et al.* (2013)
		Caretta caretta	MF	28.7-30	Wibbels (2003)
		Lepidochelys olivacea	MF	30-31	Wibbels (2003)
		Lepidochelys kempii	MF	30.2	Wibbels (2003)
		Natator depressus	MF	29.4	Stubbs *et al.* (2014)
	Dermochelyidae	*Dermochelys coriacea*	MF	29.4-29.5	Wibbels (2003)
	Chelydridae	*Chelydra serpentina*	FMF	20.3-24.2 & 25.6-28.2	Ewert *et al.* (2005)
	Emydidae	*Malaclemys terrapin*	MF	28.29	Burke and Calichio (2014)
	Geoemydidae	*Mauremys japonica*	MF	28.8	Okada *et al.* (2010)
Crocodilia	Crocodylidae	*Crocodylus acutus*	FMF	31.1 & 33.6	Charruau *et al.* (2017)
		Crocodylus acutus	FMF	31 & 32.5	Charruau (2012)
		Crocodylus johnstoni	FMF	31.5 & 32.5	Lang and Andrews (1994)
		Caiman crocodilus	FMF	31.5 & 34	Lang and Andrews (1994)
		Caiman latirostris	FMF	32-33 & 34-34.5	Marcó *et al.* (2017)
	Alligatoridae	*Alligator mississipiensis*	FMF	31.8 & 33.8	Lang and Andrews (1994)
Rhynchocephalia	Sphenodontia	*Sphenodon guntheri*	FM	22	Mitchell *et al.* (2006)

remain relatively consistent within species apart from small variations between geographically distinct sub-populations (Ewert *et al.*, 2005; Refsnider *et al.*, 2014). In contrast, pivotal temperatures vary significantly within the Testudines (Table 2.1), with freshwater turtles tending to have lower pivotal temperatures than sea turtles (Ewert *et al.*, 2005; Okada *et al.*, 2010; Burke & Calichio, 2014). Crocodilians display slightly higher and more consistent pivotal temperatures than sea turtles (Hutton, 1987; Lang & Andrews, 1994; Charruau *et al.*, 2017; Marcó *et al.*, 2017). In squamates, pivotal temperatures in species with TSD appear to vary significantly (Doody *et al.*, 2006; Gamble, 2010; Inamdar & Seshagiri, 2012), while the Rhynchocephalia display one of the lowest known pivotal temperatures among reptiles (21.6°C or 22°C, depending on subspecies) (Mitchell *et al.*, 2006).

Despite the strong influence of ambient temperature on primary sex ratios in species with TSD, developing embryos may have some control over their development. Reptile embryos can move to areas of varying temperature within the egg (Du & Shine, 2015), potentially to optimise their incubation conditions, accelerate embryonic development and expand the temperature range that produces balanced primary sex ratios (Ye *et al.*, 2019). The ability of embryos to move is limited in early and late development due to a lack of musculature and space, respectively (Shine & Du, 2018). Additionally, small eggs and eggs laid in thermally-uniform locations may lack thermal gradients large enough for embryos to utilise (Telemeco *et al.*, 2016; Shine & Du, 2018). However, if embryos are able to thermoregulate during development, they may be able to mitigate changes in ambient temperatures and maintain balanced primary sex ratios. Further studies are required to determine if an embryo's ability to move within the egg is adaptively significant i.e. are embryos capable of thermoregulation in the egg (Du & Shine, 2015; Shine & Du, 2018) or not (Telemeco *et al.*, 2016; Cordero *et al.*, 2018).

Despite the plethora of studies investigating TSD in the Reptilia, knowledge of the mechanisms of TSD remains elusive. Recent studies have found a gene, CIRBP, that is expressed differentially at male- and female-producing temperatures (Rhen & Schroeder, 2010; Rhen & Schroeder, 2017) and that differential expression of CIRBP can alter the fate of a bipotential gonad (Schroeder *et al.*, 2016). These studies have led to the development of an immunohistochemical test that can identify sex in sea turtle hatchlings (Tezak *et al.*, 2017; Tezak *et al.*, 2020). Differences in CIRBP allele frequencies may also explain why certain individuals or clutches are more likely to develop into females or males compared to other individuals (Schroeder *et al.*, 2016). Further investigation is required to fully understand why

identical temperature regimes can result in different sex ratios and how fluctuating temperatures in natural conditions determine primary sex ratios.

2.3.1.2 Locomotor performance

The effect of temperature during incubation on locomotor performance has been extensively examined in sea turtles (see review by Booth, 2017), but much less so in other reptile taxa. Locomotor performance in all reptiles appears to be optimised at intermediate incubation temperatures, with decreases in performance occurring as incubation temperature becomes more extreme in either direction (Noble *et al.*, 2017; Booth, 2018). Extended or repeated periods of high temperature during incubation consistently have negative effects on hatchling locomotor performance in all reptile species (Maulany *et al.*, 2012; Sim *et al.*, 2015). However, optimal incubation temperatures vary among and within taxa (Table 2.2).

It is important to note that many experimental studies incubate eggs within narrow temperature ranges (e.g. 3 to 4°C) or only test responses to two incubation temperatures and subsequently report linear relationships between incubation temperature and locomotor response (Booth *et al.*, 2004; Hare *et al.*, 2008). Locomotor performance in the Testudines is optimised between 26 and 30°C, with sea turtles exhibiting maximal locomotor performance at slightly higher temperatures than freshwater turtles (Burgess *et al.*, 2006; Read *et al.*, 2013). Squamates perform best at slightly lower incubation temperatures, between 24 and 28°C (Elphick & Shine, 1998; Elphick & Shine, 1999). Despite a scarcity of studies, it appears likely that crocodilians and rhynchocephalians optimise their locomotor performance at ~30°C and ~20°C, respectively, , similar to recorded pivotal temperatures (Table 2.1).

Incubation temperature has been hypothesised to impact locomotor performance by affecting embryonic muscle fibre development (Booth, 2017), affecting both the type of muscle fibres that form in embryos (Carey *et al.*, 2009), as well as fibre size (Piestun *et al.*, 2009). The response of fish embryo muscle fibre type and size to incubation temperatures varies (Blaxter, 1991; Johnston, 2006) and studies in reptiles are limited (Booth, 2018). However, the increased power production (Booth & Evans, 2011; Bell *et al.*, 2013; Sim *et al.*, 2015) and decreased stamina (Booth *et al.*, 2004; Burgess *et al.*, 2006; Ischer *et al.*, 2009) observed in sea turtle hatchlings incubated at cooler temperatures could be explained by increased fast twitch muscle fibre development and reduced size of yolk reserves in those hatchlings. Therefore, increased power production during swimming and crawling is offset by decreased stamina. Contrary findings of increased stamina of keelback snakes (*Tropidonophis mairii*) incubated at cooler temperatures (Bell *et al.*, 2013) may be explained by the brief duration of

Table 2.2: The response of various measures of locomotor performance to different incubation temperatures. The temperature at which each trait is highest is identified and temperatures where no difference in that trait was observed are separated by '&'. For studies that analysed incubation temperatures as a continuous variable, we report the range of temperatures observed and where the trait was highest, if it was highest at an intermediate temperature.

Locomotor trait	Response to incubation temperature	Incubation temperatures	Species	Reference
Powerstroke frequency	Slower at cooler temperatures	26 < 28 & 30	Green sea turtle	Booth *et al.* (2004)
		26 < 28 & 30 (2000) 25.5 < 30 (2002)	Green sea turtle	Burgess *et al.* (2006)
		Ranged from 28.5 to 32.4 A	Green sea turtle	Ischer *et al.* (2009)
Duration of time spent power stroking	Less time at cooler temperatures	25.5 < 30	Green sea turtle	Burgess *et al.* (2006)
Mean maximum thrust	More force at cooler temperatures	Warm- 30.7 Cool- 29.1 B	Green sea turtle	Booth and Evans (2011)
		Ranged from 27.9-30.9 (2010 & 2011) 31-32.6 (2012) A	Loggerhead sea turtle	Sim *et al.* (2015)
Swim speed	Faster at cooler temperatures	24.9 & 26.6 > 30.1	Freshwater keelback snake	Bell *et al.* (2013)
	Faster at warmer temperatures	26 < 29	Black ratsnake	Patterson and Blouin-Demers (2008)
Swim endurance	Higher at cooler temperatures	24.9 & 26.6 > 30.1	Freshwater keelback snake	Bell *et al.* (2013)
Crawling/running speed	Faster at warmer temperatures	20±4 < 27±4 C	Montane scincid lizard	Elphick and Shine (1998)
		16/24 < 23/31 C	Montane scincid lizard	Elphick and Shine (1999)
		18 < 22 & 26	Suter's skink	Hare *et al.* (2008)
	Faster at cooler temperatures	15/25 > 20/30 C	Striped plateau lizard	Qualls and Andrews (1999)
		24 >28 > 32 > 35	Common wall lizard	Van Damme *et al.* (1992)

		26 > 28.5 & 31	Tenerife lizard	Vanhooydonck *et al.* (2001)
		28 > 32	Kingsnake	Burger (1990)
		26 > 30 & 34	Przewalski's Toadhead Agama	Xiao-long *et al.* (2012)
		Ranged from 28.5 - 32.4	Green sea turtle	Ischer *et al.* (2009)
		Ranged from 28.1 – 32.7	Loggerhead sea turtle	Read *et al.* (2013)
		Ranged from 29.6 – 32.2	Loggerhead sea turtle	Wood *et al.* (2014)
	Faster at intermediate temperatures	Ranged from 27 -31 but highest at 29-30	Loggerhead sea turtle	Fisher *et al.* (2014)

A Incubation occurred in relocated nests on the nesting beach

B Incubation occurred in relocated nests on the nesting beach. Nests were allocated to warm or cool treatment groups with the mean temperature of those groups provided.

C Incubation occurred at fluctuating temperatures.

endurance testing (i.e. 5 minutes), potentially not long enough to differentiate between snakes with high or low endurance. The majority of tissue differentiation occurs within the first 30-40% of development and growth rates are more sensitive to temperature earlier during development than later (Andrews, 2004). Thus, it is most likely that temperature has the largest influence on reptile locomotor performance during the early stages of development. The underlying mechanisms behind temperature's effect on muscle development, and fibre type and size, is currently unknown (Booth, 2018).

In experimental studies across the Reptilia, the response of hatchling locomotor performance to incubation temperatures has varied, largely depending on the range of temperatures selected for incubation (Vanhooydonck *et al.*, 2001; Burgess *et al.*, 2006; Hare *et al.*, 2008; Patterson & Blouin-Demers, 2008). Multiple studies indicate that intermediate incubation temperatures produce hatchlings that are faster runners, crawlers and swimmers, while extreme temperatures produce slower hatchlings (Table 2.2). Future studies should focus on incubating eggs at wider ranges of temperatures (see Mueller *et al.*, 2019) that encompass the entire range of natural incubation temperatures in order to accurately identify locomotor responses to temperature. Investigations into hatchling locomotor response to temperature should also be prioritised for underrepresented taxa (i.e. Crocodilia and Rhynchocephalia).

2.3.1.3 Body size

Morphological changes (e.g. length, width, mass) in response to incubation temperature vary significantly within the Reptilia (Table 2.3). For example, in the Testudines, turtle bodies are typically longer and wider at lower incubation temperatures, but generally do not vary in mass (Gutzke & Packard, 1987a; de Souza & Vogt, 1994; Booth & Astill, 2001; Micheli-Campbell *et al.*, 2011). Conversely, squamates tend to be heavier at lower incubation temperatures (Harlow & Shine, 1999; Ji & Brana, 1999; Du & Ji, 2008; Qu *et al.*, 2011; Monasterio *et al.*, 2013; Hansson & Olsson, 2018). Measurements of squamate snout-vent length (SVL), however, vary dramatically with incubation temperature (Andrews *et al.*, 2000; Ji *et al.*, 2002; Esquerré *et al.*, 2014). In contrast to patterns observed in other taxa, crocodilian hatchling length and mass generally display almost no response to incubation temperature (Hutton, 1987; Joanen *et al.*, 1987; Allsteadt & Lang, 1995).

While short periods of extreme temperatures generally produce shorter and lighter hatchlings in sea turtles (Maulany *et al.*, 2012; Sim *et al.*, 2015), the effect of stable temperatures is

Table 2.3: The effect of incubation temperature on mass, morphology and post-hatching growth rates. Studies are allocated based on the conditions that produced the largest hatchlings and fastest growth rates.

	With warmer temperatures	With cooler temperatures	With intermediate temperature	No effect of temperature
	Increased mass			
Turtle	de Souza and Vogt (1994)	Gutzke and Packard (1987a)	Hewavisenthi *et al.* (2001); Fisher *et al.* (2014)	Janzen and Morjan (2002); Reece *et al.* (2002); Ischer *et al.* (2009); Booth and Evans (2011); Fisher *et al.* (2014); Wood *et al.* (2014)
Tortoise		Spotila *et al.* (1994)		
Snake		Du and Ji (2008); Bell *et al.* (2013)	Ji and Du (2001b)	Burger *et al.* (1987); Burger (1990)
Lizard	Elphick and Shine (1998)	Van Damme *et al.* (1992); Phillips and Packard (1994); Harlow and Shine (1999); Ji and Brana (1999); Qu *et al.* (2011); Monasterio *et al.* (2013), Hansson and Olsson (2018), Xiao-Long et al. (2012)		Qualls and Andrews (1999); Andrews *et al.* (2000); Flatt *et al.* (2001); Ji *et al.* (2002)
Crocodile			Marcó *et al.* (2010)	Hutton (1987); Webb and Cooper-Preston (1989); Allsteadt and Lang (1995)
	Increased carapace length/SVL			
Turtle		Gutzke and Packard (1987a); Reece *et al.* (2002); Booth and Evans (2011); Micheli-Campbell *et al.* (2011); Maulany *et al.* (2012); Sim *et al.* (2015)	Hewavisenthi *et al.* (2001); Fisher *et al.* (2014)	Booth and Astill (2001); Ashmore and Janzen (2003)

Snake	Burger *et al.* (1987); Burger (1990)	Bell *et al.* (2013)	Ji and Du (2001a); Ji and Du (2001b)	
Lizard	Qualls and Andrews (1999); Andrews *et al.* (2000)	Van Damme *et al.* (1992); Phillips and Packard (1994); Harlow and Shine (1999)	Ji *et al.* (2002)	Flatt *et al.* (2001); Esquerré *et al.* (2014), Hansson and Olsson (2018)
Crocodile		Hutton (1987)	Allsteadt and Lang (1995), (Marcó *et al.*, 2010)	Joanen *et al.* (1987); Webb and Cooper-Preston (1989)
	Increased carapace width			
Turtle		Booth and Evans (2011)	Hewavisenthi *et al.* (2001); Fisher *et al.* (2014)	Booth and Astill (2001)
	Increased growth rates (post-hatching)			
Turtle	Roosenburg and Kelley (1996); Janzen and Morjan (2002)	Brooks *et al.* (1991); Rhen and Lang (1995)	McKnight and Gutzke (1993)	Steyermark and Spotila (2001)
Tortoise			Spotila *et al.* (1994)	
Snake	Shine *et al.* (1997)			
Lizard	Alberts *et al.* (1997); Elphick and Shine (1999); Qualls and Andrews (1999)	Van Damme *et al.* (1992); Andrews *et al.* (2000); Esquerré *et al.* (2014)		
Crocodile	Hutton (1987)		Joanen *et al.* (1987)	

more variable, both in sea turtles (Horne et al., 2014) and other reptile taxa (Ashmore & Janzen, 2003; Du & Ji, 2006; Patterson & Blouin-Demers, 2008; Horne *et al.*, 2014).

Temperatures in underground reptile nests tend to be more stable than those at the surface (Pike *et al.*, 2010; Santidrian Tomillo *et al.*, 2017), although fluctuations could potentially occur in locations with large day- and night-time temperature differentials.

It has been suggested that temperature affects morphology by altering biochemical reactions and the resultant rate of embryonic development (Booth, 2017). Higher temperatures consistently reduce the duration of incubation (Warner *et al.*, 2011; Sim *et al.*, 2015), therefore minimising the period in which yolk can be converted into hatchling tissue and resulting in smaller hatchlings with larger residual yolk masses (Hewavisenthi *et al.*, 2001; Pan & Ji, 2001; Booth, 2006; Burgess *et al.*, 2006). However, this does not explain the contrasting morphological responses to temperature observed in the Squamata (Harlow & Shine, 1999; Qu *et al.*, 2011; Hansson & Olsson, 2018) and Testudines (Booth & Evans, 2011; Wood *et al.*, 2014). It is possible that these responses reflect differing evolutionary pressures on terrestrial (Squamata) versus largely aquatic species (Testudines). While it is presently unclear why incubation temperature does not affect crocodilian morphology, this may signal a reduced sensitivity to incubation temperatures relative to other reptile taxa (Webb & Cooper-Preston, 1989; Allsteadt & Lang, 1995).

Across the Reptilia, temperature appears to have a less consistent effect on hatchling morphology than on locomotor performance or sex determination. However, cooler incubation temperatures generally result in larger or heavier hatchlings. Further research is required to identify why taxa respond to temperature changes in different, sometimes contrasting ways and how fluctuating temperatures in natural nests may influence hatchling size.

2.3.1.4 Hatching success and development rate

Hatching success rates in the Reptilia are highest at intermediate temperatures (Table 2.4), with embryonic death occurring at extreme high or low temperatures. The optimal temperature for hatching success is slightly lower in freshwater turtles than for sea turtles (Gutzke *et al.*, 1987) and is highly variable in squamates (Burger *et al.*, 1987; Brown & Shine, 2006; Andrews, 2008). Crocodilian hatching success is highest at higher temperatures than any other taxa (Webb & Cooper-Preston, 1989; Piña *et al.*, 2003), while tuatara hatching success is highest at much lower temperatures (Thompson, 1990; Nelson *et al.*, 2004b).

Although developing embryos appear quite resilient to short-term extreme temperatures, the cumulative length of exposure has the largest effect on embryonic mortality in sea turtles (Lang & Andrews, 1994; Maulany *et al.*, 2012; Howard *et al.*, 2014; Sim *et al.*, 2015; Bladow & Milton, 2019). While thermal spikes can result in reduced hatching success (Hall & Warner, 2018), development rates are generally faster at higher temperatures for multiple taxa within the Reptilia (Hutton, 1987; Van Damme *et al.*, 1992; Du *et al.*, 2007)

The relationship between maximum hatching success and incubation temperature largely matches the one observed between pivotal temperatures and locomotor performance. Potentially of more importance than the temperature at which maximum hatching success is achieved, however, is the range of temperatures at which species can maintain high hatching success. Taxa that can develop successfully at a wide range of incubation temperatures (e.g. Pine snakes (*Pituophis melanoleucus),* Oriental garden lizards *(Calotes versicolor))* are likely to be more resilient than those that experience sharp declines in hatching success outside a narrow range (e.g. Broad-snouted caiman (*Caiman latirostris),* Beauty snake *(Elaphe taeniura)*) (Table 2.4).

2.3.2 Moisture

2.3.2.1 Sex ratio

Nest substrate moisture and humidity levels during incubation may account for some of the observed variation in hatchling primary sex ratios in species with TSD. Studies have found moisture indirectly alters nest temperatures (Lolavar & Wyneken, 2015; Sifuentes-Romero *et al.*, 2017a) and restricts oxygen availability (Cedillo-Leal *et al.*, 2017), with potentially other direct, unknown mechanisms (Lolavar & Wyneken, 2017).

Studies of both freshwater turtles (Gutzke & Paukstis, 1983; LeBlanc & Wibbels, 2009; Sifuentes-Romero *et al.*, 2017b) and sea turtles (Lolavar & Wyneken, 2015; Wyneken & Lolavar, 2015; Lolavar & Wyneken, 2017) have found that increased moisture during incubation results in increased production of male hatchlings. However, other studies have found that moisture played no role in determining primary sex ratios in certain testudines (Packard *et al.*, 1991; Bobyn & Brooks, 1994; Hewavisenthi & Parmenter, 2000) and one study in painted turtle hatchlings (*Chrysemys picata*) found that drier substrates produced more males than clutches incubated in wetter substrates (Paukstis *et al.*, 1984). However, it is difficult to compare these findings because both substrate and arrangement of the eggs differs among studies. Experiments that use vermiculite or no substrate at all, and either partially cover or separate the eggs, do not reflect natural nesting conditions. This can alter

Table 2.4: Minimum and maximum hatching success in various reptile taxa and the temperatures that produced those results.

Order	Family	Species	Maximum hatching success	Temperature	Minimum hatching success	Temperature	Reference
Crocodilia	Alligatoridae	Alligator mississipiensis	83%	32.8°C	76.20%	30.6°C	Joanen et al (1987)
		Caiman latirostris	65%	31°C	16.20%	34.5°C	Piña et al (2003)
	Crocodylidae	Crocodylus niloticus	83%	31°C	69.00%	34°C	Hutton (1987)
		Crocodylus porosus	~73%	31°C	~25%	36°C	Webb & Cooper-Preston (1989)
		Crocodylus johnstoni	63%	30°C	0%	26°C	Webb *et al.* (1983)
Rhynchocephalia	Sphenodontidae	Sphenodon punctatus	100%	21°C	87.50%	18°C	Nelson et al (2004)
		Sphenodon punctatus	62%	20°C	0%	15°C	Thompson (1990) A
Squamata	Agamidae	Calote versicolor	80.60%	27°C	3.40%	33°C	Ji et al (2002)
		Calotes versicolor	93%	27°C	53.00%	35°C	Radder et al (2002)
	Chamaeleonidae	Chamaeleo calyptratus	96.00%	25/25°C, 25/28°C & 28/28°C	86.00%	28/30°C & 30/30°C	Andrews (2008) B
		Chamaeleo chamaeleon	100%	25°C	64.40%	29°C	Díaz-Paniagua and Cuadrado (2003) C
	Colubridae	Elaphe carinata	92% (dry) & 89.5% (wet)	30°C	65.7% (dry) & 67.6% (wet)	32°C	Ji and Du (2001a) D
		Elaphe taeniura	79%	30°C	41%	32°C	Du and Ji (2008)

		Pituophis melanoleucus	99%	28°C	0.00%	21°C	Burger and Zappalorti (1988)
		Pituophis melanoleucus	97%	30°C	27.00%	21°C	Burger *et al.* (1987)
		Pituophis melanoleucus	100%	27°C	52.40%	22°C	Gutzke and Packard (1987b)
		Tropidonophis mairii	79%	24.8°C	21%	30.1°C	Brown and Shine (2006)
	Elapidae	Naja naja atra	77.1% (dry)/83.8% (wet) & 85.7% (dry)/78.7% (wet)	26°C & 30°C	30.8% (dry) & 6.8% (wet)	24°C	Ji and Du (2001b) D
	Iguanidae	Sceloporus undulatus	100%	30°C	78%	23°C & 28°C	Andrews *et al.* (2000) E
		Sceloporus undulatus	86%	30°C	0%	36°C & 38°C	Angilletta *et al.* (2000)
	Lacertidae	Podarcis muralis	73.30%	24°C	12.50%	35°C	Van Damme *et al.* (1992)
	Scincidae	Bassiana duperreyi	88%	30°C	63%	25°C	Booth *et al.* (2001) F
		Lampropholis guichenoti	70%	25°C	60%	30°C	Booth *et al.* (2000) F
Testudines	Chelidae	Elusor macrurus	89%	26°C & 29°C	56.00%	32°C	Micheli-Campbell *et al.* (2011)
	Cheloniidae	Caretta caretta	69.20%	29°C	33.30%	32°C	Fisher *et al.* (2014)
		Chelonia mydas	75%	28°C	70%	30°C	Booth *et al.* (2004)
		Chelonia mydas	80%	30°C	75.00%	26°C	Burgess *et al.* (2006) G

		Chelonia mydas	87%	25.5°C	58.30%	30°C	Burgess *et al.* (2006) F, H
		Chelonia mydas	71%	27.6°C	40.00%	30°C	Godfrey and Mrosovsky, (2006)
		Eretmochelys imbricata	80-100%	28°C & 29.5°C	40-80%	32.5°C	Dobbs *et al.* (2010)
	Emydidae	Chrysemys picta	83%	26°C	77.00%	30°C	Janzen and Morjan (2002) I
		Emydoidea blandingii	95%	26.5°C	0.00%	22°C	Gutzke and Packard (1987b)
	Testudinoidea	Gopherus agassizii	96%	28.1°C	29.00%	35.3°C	Spotila et al (1994) J
	Trionychidae	Pelodiscus sinensis	97%	27°C	44.00%	23°C	Du and Ji (2003)
		Pelodiscus sinensis	96.60%	28°C	68%	34°C	Ji et al (2003)

A Incubation treatments included moisture treatments. Calculation of hatching success for each temperature was the average of the moisture treatments at that temperature.

B Incubation temperatures were changed midway through incubation. Treatment groups were combined for analysis (25/25, 25/28 & 28/28 vs. 28/30 & 30/30).

C Only two incubation temperatures (25°C and 29°C.)

D Each temperature split into dry (-220kPa) and wet (0kPa) moisture treatments.

E Mortality was very low (8.4%) in all treatments

F Only two incubation temperatures- 25°C and 30°C

G 2000 experiments

H 2002 experiments

I Only two incubation temperatures: 26°C and 30°C

J Only 0.4% moisture treatments included here

evaporative rates and moisture dynamics around the eggs, potentially influencing the response of the developing embryos to moisture. Additionally, studies differ in their measures of moisture, with some reporting water potential (kPa) while others report water concentration (%), which can be measured as weight/weight (w/w) or volume/volume (v/v). These inconsistencies make quantitative comparisons difficult.

Further, temperature and moisture strongly interact (Hill *et al.*, 2015), making it difficult to isolate their individual effects on sex determination. Lolavar and Wyneken (2017) attempted to do this with sea turtle embryos by controlling evaporative cooling rates and maintaining all treatments at the same temperature. They found that nests subjected to evaporative cooling produced more males than nests that minimised evaporative cooling. Interestingly, all of the high moisture treatments in this study produced fewer females, irrespective of evaporative cooling rates, than would be expected based on temperature alone. A potential cause of this difference is that surface and internal egg temperatures are similar, but the difference between egg and air temperatures can be as high as 2°C in sea turtle nests depending on humidity (Tezak *et al.*, 2018). Thus, incubator air temperature measured in Lolavar and Wyneken (2017) may have been higher than the internal egg temperature, resulting in higher than expected male hatchling production.

Overall, the role of moisture in influencing reptile primary sex ratios is not clearly defined. Research has been biased toward investigations in the Testudines, with comparison among studies difficult due to differences in experimental conditions (e.g. egg arrangement, substrate type) and reported measurements of moisture. Further research is required to identify whether moisture can directly influence primary sex ratios and if so, to identify the mechanism. It is currently thought that the interaction between moisture and temperature has the largest effect on sex determination (Sifuentes-Romero *et al.*, 2017b), highlighting the importance of considering multiple environmental variables when investigating the effects of incubation conditions on hatchling phenotypes. Investigations into the effect of moisture during incubation are also recommended for other reptile taxa (i.e. non-Testudines).

2.3.2.2 Locomotor performance

The majority of research on possible effects of moisture during incubation on locomotor performance has involved snapping turtles (*Chelydra serpentina*). Hatchlings incubated in wet conditions are generally faster swimmers and crawlers (Miller *et al.*, 1987; Miller, 1993; Finkler, 1999) and also show a smaller reduction in crawling speed after desiccation compared to hatchlings incubated in dry conditions (Finkler, 1999). There are few studies

outside the Testudines, but contrasting responses exhibited in lizards (Squamata) may reflect habitat-specific adaptations. In tropics-dwelling keelback snakes (*Tropidonophis mairii)*, hatchlings produce more contractive force when incubated at higher moisture levels than those incubated at lower moisture levels (Brown & Shine, 2006). In contrast, squamates from more arid zones display no change in performance at different moisture levels during incubation (Flatt *et al.*, 2001; Warner & Andrews, 2002; Du & Shine, 2008).

There are several possible explanations for improved locomotor performance of some reptile hatchlings incubated in wet conditions. The first is that better performance is a result of the hatchling's larger size (Miller, 1993), although this is not always the case (Du & Shine, 2008). Another possibility is that hatchlings incubated in wetter conditions accumulate lactate more slowly than hatchlings incubated on or within dry substrates (Miller *et al.*, 1987). Hatchlings incubated in dry environments have larger residual yolk mass relative to their body mass (Christian *et al.*, 1991), and may require increased anaerobic energy expenditure to carry this additional yolk mass that is not contributing to locomotion (Miller *et al.*, 1987). However, these hatchlings with larger yolk reserves will also have access to greater energy reserves when moving this mass (Radder *et al.*, 2004). Lastly, moisture may directly or indirectly influence embryonic muscle development, but the mechanisms behind these potential effects are unknown.

Although reptile hatchlings incubated in wetter conditions are generally stronger and faster than hatchlings incubated in dry conditions, studies have been biased toward the Testudines and further investigation is recommended for other reptile taxa. Hypotheses for direct and indirect moisture-dependent effects on locomotor performance require further testing.

2.3.2.3 Body size

Increases in moisture during incubation result in the production of heavier and longer hatchlings in freshwater and sea turtles (Gutzke *et al.*, 1987; Finkler, 1999; Hewavisenthi *et al.*, 2001; Reece *et al.*, 2002; Bodensteiner *et al.*, 2015), snakes (Brown & Shine, 2006; Brown & Shine, 2018) and lizards (Phillips & Packard, 1994; Marco *et al.*, 2004; Du & Shine, 2008; Xiao-long *et al.*, 2012). Studies on the effects of moisture on crocodilian hatchlings are lacking, but as crocodilian eggshells are largely resistant to water uptake or loss, the response of embryos to moisture changes are likely to be limited (Ferguson, 1981; Packard *et al.*, 1982).

Increased moisture levels in sea turtle nests during incubation results in hatchlings converting more yolk mass into body mass, thus hatching at a larger size (Miller *et al.*, 1987; Christian *et*

al., 1991; Hewavisenthi *et al.*, 2001). However, the mechanisms behind this remain unknown. One possible explanation is that drier incubation conditions cause higher blood viscosity in the developing embryo, reducing the rate at which nutrients can be converted into body mass (Packard & Packard, 1986; Packard & Packard, 1989). However, Bilinski *et al.* (2001) found that calcium mobilisation from eggshell to embryo in leatherback turtle (*Dermochelys coriacea*) embryos was higher in drier incubation conditions. Additionally, McGehee (1990) found that carapace length in loggerhead turtle (*Caretta caretta*) hatchlings decreased with increasing moisture levels from 0% w/w water concentration to 24% w/w concentration. Sea turtle nests are typically in the 2-5% w/w range (Wood *et al.*, 2000), so it is possible that the moisture levels used by McGehee (1990) were too high, resulting in reduced embryonic growth and smaller hatchling size. Indeed, very high moisture is often associated with reduced hatching success in loggerhead turtles (Foley *et al.*, 2006). Incubation moisture levels do not generally influence post-hatching growth rates in either testudines (Brooks *et al.*, 1991; McKnight & Gutzke, 1993; Bobyn & Brooks, 1994; Bodensteiner *et al.*, 2015) or squamates (Alberts *et al.*, 1997; Warner & Andrews, 2002; Tang *et al.*, 2012). However, some studies have observed faster post-hatching growth rates in sea turtle hatchlings incubated in wetter conditions (Erb *et al.*, 2018), suggesting that further studies are required.

Embryos are generally less sensitive to moisture than they are to temperature (Packard *et al.*, 1989a; Flatt *et al.*, 2001; Xiao-long *et al.*, 2012). Optimal moisture levels appear to produce larger and heavier hatchlings, but extreme moisture levels can have negative effects on body size and growth. Low moisture levels potentially increase embryo blood viscosity to levels that limit the mobilisation of nutrients and oxygen and thus reduce hatchling body size (Packard, 1991). However, future research should ensure that experimental moisture levels cover a wide enough range to capture potential responses, as only moisture levels above or below critical levels may impact tissue development via yolk mobilisation (Hewavisenthi *et al.*, 2001) or blood viscosity (Packard, 1991). Research on moisture concentrations during incubation should consider standardising measures of moisture within and around nests, in order to facilitate comparisons among studies.

2.3.2.4 Hatching success and development rate

Excess moisture or inundation during incubation can result in decreased hatching success or even loss of the entire clutch (Kofron, 1989; Villamarín-Jurado & Suárez, 2007; Caut *et al.*, 2010). While reptile eggs can be quite resistant to brief or intermittent inundation from

rainfall, river flooding or unusually high tides (Caut *et al.*, 2010; Pike *et al.*, 2015; Cedillo-Leal *et al.*, 2017), repeated stress due to excessive moisture almost always leads to embryonic mortality (Foley *et al.*, 2006). Hatching success after rainfall or flooding varies depending on the elevation of egg clutches within a landscape (Kraemer & Bell, 1980; Kushlan & Jacobsen, 1990) and the stage of embryonic development (Cedillo-Leal *et al.*, 2017; Rafferty *et al.*, 2017). Inundation appears to limit oxygen supply to the developing embryos such that late stage embryos, with higher metabolic demands, are more sensitive to oxygen deprivation than early stage embryos (Cedillo-Leal *et al.*, 2017).

Hatching success varies significantly among taxa but is generally greatest at intermediate moisture levels (Packard *et al.*, 1991; Foley *et al.*, 2006; Marco & Díaz-Paniagua, 2008). Species-specific differences in sensitivity to moisture concentrations likely reflect their adaptation to surrounding environmental conditions. For example, desert tortoises (*Gopherus agassizii*) have maximum hatching success in drier substrates (Spotila *et al.*, 1994), while painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*) experience highest hatching success in much wetter conditions (Paukstis *et al.*, 1984; Packard *et al.*, 1987; Packard *et al.*, 1989b; Packard *et al.*, 1991). Thus, each species' hatching success is maximised in their respective habitats i.e. dry and wet. These contrasting responses to moisture during incubation may be attributable to differences in permeability between rigid and soft-shelled turtle eggs (Packard *et al.*, 1999; Booth, 2002). In contrast to observed patterns in the Testudines, multiple squamate species show no differences in hatching success following changes in moisture during incubation (Phillips *et al.*, 1990; Flatt *et al.*, 2001; Ji & Du, 2001a).

Overall, eggs incubated in dry conditions generally hatch earlier than those in wet conditions (McGehee, 1990; Packard *et al.*, 1991; Miller, 1993; Flatt *et al.*, 2001) and moisture appears to affect hatching success, at least in species with soft-shelled eggs (e.g. Rhynchocephalia, most squamates). Reptile embryos are generally resistant to intermittent periods of extreme high or low moisture, however extended or regular exposure to very wet or very dry conditions considerably reduces hatching success. Future research should investigate taxa-specific responses to moisture during incubation, noting that habitat preferences and egg types likely influence these responses.

2.3.3 Oxygen concentration

Diffusion is the main driver of oxygen into reptile eggs. In clutches laid above ground, oxygen quickly diffuses into the egg, while in underground nests oxygen must first diffuse

through the substrate along a concentration gradient (Prange & Ackerman, 1974; Hillel, 2003). As a result, oxygen concentrations within underground nests are influenced by a number of physical factors (e.g. depth, moisture content, temperature) and characteristics of the nest substrate (e.g. grain size, rugosity, pore spacing) (Ackerman, 1980; Lutz & Dunbar-Cooper, 1984; Christian & Lawrence, 1991; Ryberg & Fitzgerald, 2015). Similarly, oxygen availability can be reduced due to surrounding biotic influences (e.g. proximity to other nests, clutch size, microbes or organic material) and increased metabolic demands of embryos at later stages of development (Ackerman, 1980; Lutz & Dunbar-Cooper, 1984; Christian & Lawrence, 1991; Bézy *et al.*, 2015).

Some reptile species are able to arrest embryonic development in response to reduced oxygen levels. In freshwater and sea turtles, low oxygen levels (~1%) within the oviducts allow females to arrest the embryonic development of eggs until oviposition (Rafferty *et al.*, 2013; Williamson *et al.*, 2017b). However, once embryonic development has commenced, embryos require a relatively constant supply of oxygen and cannot re-arrest if exposed to hypoxic conditions (Williamson *et al.* (2017b). Unlike sea turtle embryos, crocodilians do not appear capable of extending embryonic arrest post-oviposition (Williamson *et al.*, 2017a).

2.3.3.1 Sex ratio

Research on the effect of oxygen concentration on sex determination in the Reptilia is limited. Studies in the Testudines (Etchberger *et al.*, 1991) and crocodilians (Deeming & Ferguson, 1991) have found no relationship between oxygen concentration during incubation and sex determination. Further research is required to discover if the same is true in squamates and tuataras.

2.3.3.2 Locomotor performance

The effect of oxygen concentration during incubation on locomotor performance is complex and varies across the Reptilia. Chinese soft-shelled turtles (*Pelodiscus sinensis*) maintained at 22% oxygen for the entirety of incubation were faster crawlers compared to hatchlings incubated at 12% or 30% oxygen (Liang *et al.*, 2015). However, this effect was observed at very high incubation temperatures of 34°C, but not at 26.5°C. The effect of oxygen concentration may have been greater at 34°C than at 26.5°C because of the increased metabolic demands of embryos at higher temperatures. Additionally, hatchlings incubated in hyperoxia did not exhibit improved locomotor performance compared to those incubated at normoxia. In contrast, flatback sea turtle (*Natator depressus*) hatchlings incubated in

hyperoxic air (42% oxygen) for the first 5 days followed by normoxia for the remainder of incubation were faster crawlers but slower swimmers than hatchlings incubated entirely at normoxia (21% oxygen), though the long-term fitness advantages of hyperoxia remain unclear (Rings *et al.*, 2014). In Mongolian racerunner lizards (*Eremias argus*), oxygen concentrations during incubation did not influence sprint speed or hatchling size (Sun *et al.*, 2014; Li *et al.*, 2020).

2.3.3.3 Body size

Higher concentrations of oxygen during incubation generally result in larger hatchlings in all reptile taxa, while lower oxygen concentrations likely limit the metabolism of embryos, resulting in reduced conversion of yolk into hatchling mass (Etchberger *et al.*, 1991; Warburton *et al.*, 1995; Liang *et al.*, 2015; Parker & Dimkovikj, 2019).

2.3.3.4 Hatching success and development rate

Reptile embryos become more susceptible to hypoxia-induced mortality as they develop (Andrews *et al.*, 2000; Booth, 2000; Cedillo-Leal *et al.*, 2017; Cordero *et al.*, 2017). Even a few hours of hypoxia can reduce hatching success (Pike *et al.*, 2015), as can maintaining embryos in hypoxia-induced arrest for extended periods (Rafferty *et al.*, 2013). It is unlikely that developing embryos experience hyperoxia (i.e. atmospheric oxygen tensions above 21%) under natural conditions. However, studies have shown that hyperoxia does not generally result in higher hatching success compared to normoxia (~21%) (Etchberger *et al.*, 1991; Rings *et al.*, 2014; Sun *et al.*, 2014; Liang *et al.*, 2015; Li *et al.*, 2020). Therefore, increasing oxygen concentration does not appear to be a viable way of increasing hatching success in species with high embryonic mortality, such as leatherback sea turtles. Oxygen concentrations in the centre of underground nests are generally lower than those on the periphery (Wallace *et al.*, 2004), resulting in reduced hatching success in eggs in the centre of the nest compared to the periphery (Ralph *et al.*, 2005).

2.3.3.5 The role of carbon dioxide

When considering the factors that limit oxygen supply to developing embryos, it is also important to consider the removal of carbon dioxide. Generally, factors that limit oxygen supply also limit carbon dioxide removal, which in buried nests, leads to reduced oxygen concentrations near the centre of egg clutches (Ralph *et al.*, 2005) and increased carbon dioxide levels (Christian & Lawrence, 1991; Ackerman *et al.*, 1997). Although studies that

control oxygen concentration while manipulating carbon dioxide concentrations are limited, laboratory research on freshwater turtles has shown that higher carbon dioxide levels (10-15%) result in female-biased sex ratios, longer incubation durations and smaller hatchlings with larger residual yolks compared to low carbon dioxide levels (0-5%) (Etchberger *et al.*, 1992; Ewert *et al.*, 2002). In natural nests, embryonic carbon dioxide production (Booth, 2000) and concentrations around the eggs increase throughout incubation (Lutz & Dunbar-Cooper, 1984). For example, broad-shelled river turtle (*Chelodina expansa*) embryos are able to tolerate periods of hypercapnia (~6.7kPa) for several successive days (Booth, 1998), and hatching success in leatherback sea turtles is highest at ~6kPa carbon dioxide (Garrett *et al.*, 2010). Carbon dioxide levels in natural nests are generally around 2kPa, though periods of rain result in elevated carbon dioxide levels and carbon dioxide levels increase during incubation (Booth, 1998). Species that lay their eggs above ground are less likely to experience elevated carbon dioxide levels because the diffusion of gases is not impeded by substrate.

The effect of oxygen concentration on embryonic development and hatchling phenotypes has been relatively unstudied in comparison to the effects of temperature and moisture but oxygen concentration has important implications for successful embryonic development, hatchling size and locomotor performance. It also appears to have strong interactions with both temperature and moisture that require further investigation. Carbon dioxide has also been shown to influence hatchling phenotypes, most notably hatchling sex as well as hatching success (Etchberger *et al.*, 1992; Booth, 1998; Ewert *et al.*, 2002). However, studies on the effect of carbon dioxide on other phenotypes such as locomotor performance are limited. The lack of a relationship between oxygen concentration and sex determination suggests that carbon dioxide may directly influence hatchling phenotypes rather than indirectly by limiting oxygen availability to developing embryos. More studies on the role of oxygen and carbon dioxide during incubation are required, particularly in squamates and crocodilians.

2.3.4 Salinity

Elevated salinity is becoming increasingly concerning in terrestrial, freshwater and marine ecosystems (Nielsen *et al.*, 2003; Pachauri *et al.*, 2014) because of sea level rise and anthropogenic activities such as mining and agriculture (Cañedo-Argüelles *et al.*, 2013; Kaushal *et al.*, 2018). Increases in salinity usually decrease hatching success in turtles (Bustard & Greenham, 1968; Foley *et al.*, 2006; Bower *et al.*, 2013) and crocodilians

(Mazzotti, 1989), although this is not always the case (Bézy *et al.*, 2015). Hatchlings tend to be smaller when incubated in substrates with higher salinities compared to less saline environments, displaying phenotypes that are similar to those seen at low water potentials i.e., dry conditions (Bower *et al.*, 2013; Bézy *et al.*, 2015). It is possible that regulating and removing excess salts requires considerable energy and reduces the energy available for growth (Holliday *et al.*, 2009). Similarly, American crocodile (*Crocodylus acutus*) eggs sprayed with seawater had lower egg mass, while eggs sprayed with fresh water increased in mass (Bustard & Greenham, 1968), perhaps indicating that increased salinity interferes with normal egg metabolism and/or osmotic gradients outside of the egg.

Salinity appears to have the opposite effect to moisture on reptile embryos, appearing to cause low hatching success and decreased hatchling size under high salinity conditions. Further, embryo and hatchling traits seem to be less sensitive to changes in salinity than changes in temperature. Further research is needed to elucidate the effects and mechanisms of salinity on hatchling phenotypes across the Reptilia.

2.4 THE IMPORTANCE OF MONITORING INTERACTIONS AMONG ENVIRONMENTAL FACTORS

Studies investigating how hatchling phenotypes are impacted by incubation conditions typically manipulate or test a single environmental factor. However, all aspects of weather and climate are interconnected and change in a single factor without concomitant changes in one or more other factors is unlikely in the natural setting. Variation in even a single environmental factor will therefore likely result in multiple alterations to incubation conditions that may vary among individual clutches. However, probably for reasons of simplicity and practicality, few studies investigate how simultaneous changes in multiple environmental factors may influence one another and subsequently affect hatchling phenotypes. Here we discuss the need to consider multiple environmental variables and assert that this approach provides a more sophisticated understanding of how incubation conditions influence hatchling traits.

2.4.1 How do environmental factors influence one another?

In broad terms, incubation conditions are largely driven by the surrounding climate. However, finer-scale variation in incubation conditions arises due to the presence and interaction of multiple environmental factors, such as temperature, moisture, gas concentrations, salinity, and properties of the nest substrate.

Temperature and moisture are the main determinants of incubation conditions within clutches of eggs (Table 2.5; figure 2.1), and this combination is accordingly the most studied. Both factors influence each other and also have measurable effects on oxygen concentration and salinity (Lutz & Dunbar-Cooper, 1984; Ackerman *et al.*, 1997; Foley *et al.*, 2006; Chen *et al.*, 2010). Warmer ambient air temperatures drive increases in nest temperatures, but also increase evaporation rates, resulting in lower nest moisture levels (Ackerman *et al.*, 1997; Shine *et al.*, 2002). Conversely, moisture concentrations increase with rainfall and proximity to water sources, generally reducing incubation temperatures (Webb *et al.*, 1977; Houghton *et al.*, 2007; Warner & Shine, 2008b; Charruau, 2012; Tezak *et al.*, 2018). Water flowing through the nest substrate can mobilise salts and other water-soluble minerals (Mazzotti *et al.*, 1988; Ackerman *et al.*, 1997), while saline water sources (e.g. tidal over-wash) can deposit salts around underground clutches as the water evaporates (Foley *et al.*, 2006). Oxygen concentrations within underground nests are largely determined by the substrate type, moisture levels and the metabolic needs of the developing embryos in the nest and any adjacent nests (Ackerman *et al.*, 1997; Hillel, 2003). The effect of oxygen concentration and salinity on moisture, temperature or each other is limited (Table 2.5: figure 2.1), but salt concentrations can influence moisture availability in sea turtle nests (Ackerman *et al.*, 1997). Experiments in reptile taxa have shown that both salt and oxygen concentrations can influence developing embryo's responses to temperature and moisture (Bustard & Greenham, 1968; Liang *et al.*, 2015; Parker & Dimkovikj, 2019).

Nests laid above ground directly exposed to air are less likely to experience hypoxic conditions but are more susceptible to changes in humidity and experience greater thermal variation (Seymour & Ackerman, 1980; Booth, 2006). For underground nests, incubation conditions are strongly influenced by the characteristics of the substrate (Mortimer, 1990; Mitchell & Janzen, 2019). Large grain sizes with large spaces between grains allow water and gases to flow more easily than substrates with small grain sizes (Foley *et al.*, 2006). However, depending on shape, larger particle diameters generally result in decreased total pore space compared to fine-grained substrates and the resulting decrease in total pore space leads to decreased water content around nests in coarse soils or sands (Hillel, 2003). Particle size also may affect the diffusion of gases and the conduction of heat around the nest. Substrates with greater moisture content are generally better conductors of heat than dry substrates, but are less permeable to gases (Hillel, 2003) and are more likely to experience evaporative cooling. Differences among substrate types therefore alter the nest microclimate relative to the broader external environment. It is important to note that although substrate

Table 2.5: The interacting effects of environmental variables within reptile nests. For salinity and oxygen concentration, we also list how they can modulate the response of developing embryos to other environmental variables.

	Temperature	**Moisture**	**Oxygen concentration**	**Salinity**
Increased temperature		Increased evaporative rates resulting in reduced nest moisture levels [A]	Nest temperature generally increases during incubation due to metabolic heat production of the embryos. Both the increased temperatures and the increased development and size of the hatchlings results in increased oxygen demands for the embryos and results in decreased oxygen availability within the nest [B] Temperature can also influence diffusion rates and gas densities within clutches [C]	Increased temperatures do not directly influence salt concentration within nests, but increased temperatures can increase evaporative rates resulting in increased salt concentration within nests [A]
Increased moisture	Decreased temperature either via direct cooling or increased evaporative cooling [A,E,F]		Water displaces air in-between substrate particles resulting in reduced oxygen availability within the nest [A,I,J]	Depends on the salinity of the water. Seawater can deposit salts while rainfall can rinse the nest thereby reducing salinity [A,K]
Increased oxygen concentration	Oxygen concentration does not directly influence nest temperatures, but higher oxygen levels can help embryos be more resistant to thermal stress compared to embryos developing in low oxygen environments [D,L]	Oxygen concentration does not directly influence nest moisture but caiman embryos that had access to oxygen via air bubbles trapped on their rough shell had increased resilience to inundation compared to embryos with smooth shells [G]		Oxygen concentration does not influence salt concentration
Increased salinity	Salinity does not influence nest temperatures.	Salt concentrations can influence water gradients and potential within nests. However, the effects of salt on the movement of water within nests is minimal [A]	Salinity does not directly influence oxygen concentrations within nests. However, increased salinity can result in increased metabolic stress for developing embryos. This can impact embryonic metabolic rates and the availability of oxygen within the nest [H]	

[A] Ackerman *et al.* (1997)
[B] Chen *et al.* (2010)
[C] Ackerman (1980)
[D] Liang *et al.* (2015)
[E] Houghton *et al.* (2007)
[F] Tezak *et al.* (2018)
[G] Cedillo-Leal *et al.* (2017)
[H] Bustard and Greenham (1968)
[I] Caut *et al.* (2010)
[J] Kam (1994)
[K] Foley *et al.* (2006)
[L] Smith *et al.* (2015)

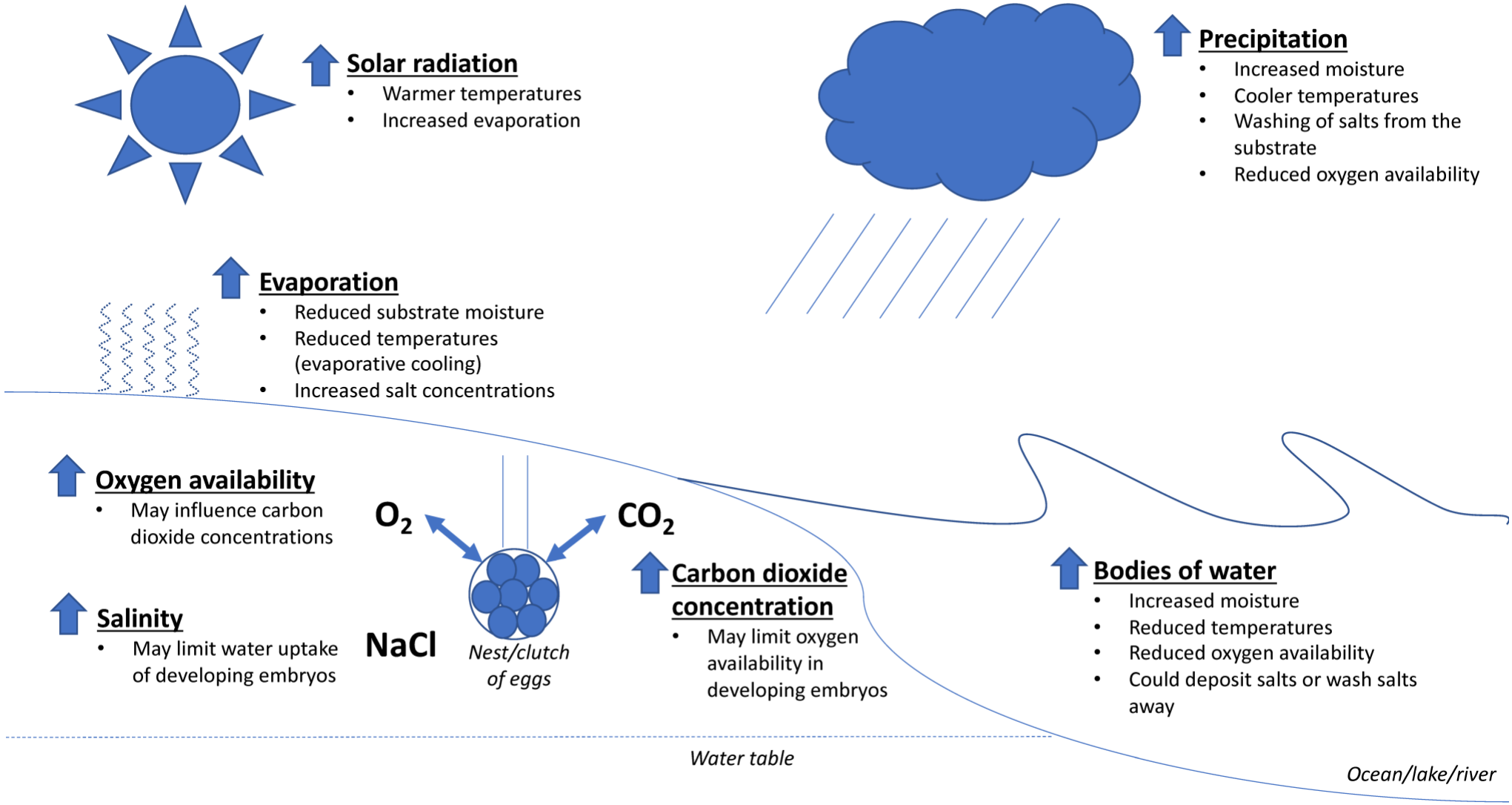

Figure 2.1: A diagrammatic representation of how environmental variables interact and influence nest conditions. Bodies of water represents both above ground and underground water sources such as oceans, lakes, rivers and the water table. It also refers to areas such as valleys where water can collect and pool. The listed responses to bodies of water represents the likely changes to environmental variables as a nest becomes closer to that body of water.

type can alter the nest microclimate, this does not guarantee that hatchling traits will also change (Stewart *et al.*, 2019).

Studies of single environmental variables are vital for understanding how specific factors influence hatchling phenotypes under controlled conditions. However, as attention shifts from controlled experiments to understanding incubation conditions *in situ*, more research is needed to identify the effects of interacting environmental factors. This not only includes understanding how environmental factors influence one another, but also investigating how changes in one factor can influence an embryo's subsequent response to a different factor. This information would improve current models of hatchling phenotypic variation, which in turn would provide a clearer and more accurate understanding of which combinations of environmental variables maximise reproductive fitness in adults than what is currently available.

2.5 WHAT ARE THE IMPLICATIONS OF ALTERED INCUBATION CONDITIONS FOR REPTILE POPULATIONS?

2.5.1 How might climate change affect hatchling phenotypes?

Reptile hatchlings are predicted to become smaller, lighter and generally less capable of survival under anthropogenic climate change, largely because of increased air and incubation temperatures. Small hatchlings that emerge with large yolk reserves may have greater endurance than large hatchlings with small yolk reserves, but in the case of sea turtles, these modest increases in endurance will not be enough to overcome reduced swimming speeds in predator-dense coastal waters and an inability to escape wave zones or currents (Cavallo *et al.*, 2015). In squamates, warming incubation temperatures under climate change are likely to result in slower runners (Burger, 1990; Van Damme *et al.*, 1992; Qualls & Andrews, 1999; Vanhooydonck *et al.*, 2001; Xiao-long *et al.*, 2012) that are less capable of escaping predation than hatchlings currently being produced (Warner & Andrews, 2002; Husak, 2006b; Husak, 2006a). Despite the negative effects of warmer incubation temperatures during embryonic development on locomotor performance (Bell *et al.*, 2013; Sim *et al.*, 2015), warmer ambient air and water temperatures may actually boost hatchling reptile locomotor performance (Christian & Tracy, 1981; Chen *et al.*, 2003; Booth & Evans, 2011) because ambient temperature also influences reptile locomotor performance (Booth & Evans, 2011; Aidam *et al.*, 2013). However, changes to incubation temperatures are likely to have a greater effect on hatchling phenotypes than ambient temperatures post-hatching because the ability of embryos to thermoregulate is limited (Telemeco *et al.*, 2016; Cordero *et al.*, 2018).

Expected increases in storm intensity (Pachauri *et al.*, 2014), including extended deluges, are likely to decrease reptile hatching success because of flooding and submersion of eggs (Kam, 1994). Small increases to moisture caused by increased rainfall may have positive effects for hatching success in some reptile species by reducing incubation temperatures and by increasing evaporative cooling (Houghton *et al.*, 2007; Warner & Shine, 2008b; Charruau, 2012). Conversely, a decrease in rainfall may further exacerbate the effects of increased temperatures on hatching success in the Reptilia. Like hatching success, both hatchling body size and locomotor performance will benefit from small increases in moisture levels during incubation (Miller *et al.*, 1987; Díaz-Paniagua & Cuadrado, 2003). However, decreases in moisture or anomalously high moisture levels (e.g. flooding or extreme rainfall events) will have negative consequences for hatchling development and survival.

Under current predictions of climate change (Pachauri *et al.*, 2014; Hoegh-Guldberg *et al.*, 2018) ,increases in air and nest temperatures compared to current conditions are likely to alter primary sex ratios, reduce hatching success and produce smaller, weaker hatchlings in most reptile taxa (Santidrián Tomillo *et al.*, 2012; Santidrián Tomillo *et al.*, 2015; Booth, 2018). Changes to moisture levels are expected to vary globally, with moderate rainfall increases mitigating some of the effects of increased temperatures in some regions. Hatching success is expected to decrease as extreme rainfall, flooding, storm surges and sea level rise reduce oxygen availability to developing embryos. Sea level rise on nesting beaches and land clearing in terrestrial nesting sites, combined with increased evaporation rates, may increase salinisation of incubation sites, leading to decreased hatching success and hatchling size. For marine reptiles, beach nourishment to combat coastal erosion may reduce hatching and emergence success, depending on the activities and techniques used, timing of construction and the quantity and quality (i.e. grain size, sorting, albedo and conduction) of the nourishment material used to replace lost sand (Grain *et al.*, 1995; Speybroeck *et al.*, 2006; Lutcavage *et al.*, 2017). However, beach nourishment generally results in reduced hatching success in sea turtles (Caderas, 2016; Cisneros *et al.*, 2017). Reptile responses to these changes are likely to vary based on physiological differences such as the permeability of the eggshell (Packard *et al.*, 1982), the ability of species to alter where they lay their eggs (Kamel & Mrosovsky, 2004; Warner & Shine, 2008b) and their nesting phenology (Neeman *et al.*, 2015).

Environmental variation, as a result of climate change, may not only influence hatchling phenotypes by altering incubation conditions, it is also likely to alter maternal effects on hatchling phenotypes. Altered environmental conditions can influence maternal nutrition,

body condition and thermoregulation, resulting in altered allocation of resources to embryos and altered nesting behaviour (Ma et al., 2014, Telemeco et al., 2010, Warner, 2014, Price et al., 2004). Many studies, mostly in squamates, compare the relative effects of maternal investment and incubation conditions on hatchling phenotypes. However, the relative influence of maternal effects and incubation conditions varies between species and even populations. Incubation duration is largely controlled by incubation conditions, and mass by maternal effects, while the response of hatchling morphometrics varies (Du et al., 2010, Lu et al., 2014, Qualls and Shine, 1998). In some cases, the thermal regimes experienced by mothers can influence the body size and thermal preferences of offspring (So and Schwanz, 2018). Further research is required to identify the relative influence that altered environmental conditions have on offspring phenotypes directly during incubation and indirectly by altering maternal investment to reproduction. Particular attention should be given to how differences in maternal investment influence the phenotypic responses of offspring and the consequences for population viability.

2.5.2 What are the consequences for population viability?

Studies on the effects of climate-mediated changes in incubation conditions have generally focused on primary sex ratios and their long-term consequences for adult populations (Telemeco *et al.*, 2009; Fuentes *et al.*, 2010; Mitchell *et al.*, 2010; Telemeco *et al.*, 2013; Hays *et al.*, 2017). In the short term, climatic variation is unlikely to have significant effects on adult populations because environmental fluctuations tend to cancel each other out (Godfrey *et al.*, 1996) over the lifespans of most reptile taxa. Additionally, many reptile populations are likely to be somewhat resilient to biased primary and adult sex ratios, subject to a growth trade-off (i.e. feminisation increasing population growth rates until collapsing due to a lack of males) (Wapstra *et al.*, 2009; Boyle *et al.*, 2014; Hays *et al.*, 2017; Laloë *et al.*, 2017). For instance, in sea turtles, differences in breeding periodicity between the sexes can balance operational sex ratios despite biased adult sex ratios (Hays *et al.*, 2010; Hays *et al.*, 2014). However, projected long-term increases in global temperatures (Pachauri *et al.*, 2014; Hoegh-Guldberg *et al.*, 2018) are likely to result in increased production of one sex (i.e. males for FM species or females for MF and FMF species), resulting in unbalanced adult sex ratios and the risk of eventual population collapse (Mitchell *et al.*, 2010; Santidrián Tomillo *et al.*, 2014; Hays *et al.*, 2017).

Further, sex-specific differences in survival rates can significantly alter the sex ratios of hatchlings recruited into adult populations (Steen *et al.*, 2006; Grüebler *et al.*, 2008). Generally, males and females from the same clutch do not differ in their locomotor performance or morphology (Booth *et al.*, 2004; Marcó *et al.*, 2010). However, variation in hatchling traits among clutches can alter hatchling recruitment in a sex-specific manner (Figure 2.2). For example, cool and wet incubation conditions may result in a male-biased clutch of larger and faster hatchlings, while warm and dry incubation conditions may result in a female-biased clutch of smaller and slower hatchlings (Rivas *et al.*, 2019). The larger and faster male-biased clutch may be more capable of chasing prey and escaping predators, and thus more likely to experience greater survival rates than the female-biased clutch (Civantos & Forsman, 2000; Gyuris, 2000; Santidrián Tomillo *et al.*, 2014). Thus, in this scenario more males are likely to survive and be recruited into the adult population, even if the primary sex ratio of the two nests combined was approximately 1:1. It is possible that sex-specific survival rates (and thus sex ratios) may vary among life stages, but more cross-taxa research is needed to confirm this.

It is important to note that climate effects on sex ratios are likely to be non-uniform and may even benefit certain taxa. For instance, reptile populations at higher latitudes may produce more balanced sex ratios and greater reproductive output under climate change (Kallimanis, 2010). Similarly, in a generally warmer climate, weather events such as protracted periods of rainfall may become important in boosting hatching quality and increasing production of the less common sex in species with TSD (Houghton *et al.*, 2007). Gravid females may gain a reproductive benefit by laying their eggs during periods of the breeding season that produce higher-quality hatchlings, or hatchlings of the less-common sex (Shine & Harlow, 1996; Löwenborg *et al.*, 2011). Individuals or sub-populations that produce hatchlings of the less-common sex will become more valuable for maintaining population viability (Baptistotte *et al.*, 1999; Stubbs *et al.*, 2014) because of their ability to balance sex ratios at the population level (Bowen *et al.*, 2005). Research to identify these valuable populations and maximise the production of the less-common sex should be prioritised.

Despite the importance of sex ratios, reductions in hatching success may have a larger effect on population viability. Embryonic mortality appears likely to impact population viability in squamates and Chelonians, potentially even before incubation conditions become extreme enough to substantially alter adult sex ratios (Santidrián Tomillo *et al.*, 2012; Santidrián Tomillo *et al.*, 2014; Hays *et al.*, 2017; Laloë *et al.*, 2017; Carlo *et al.*, 2018). Higher incubation temperatures are expected to cause this increase in mortality, but variation in other

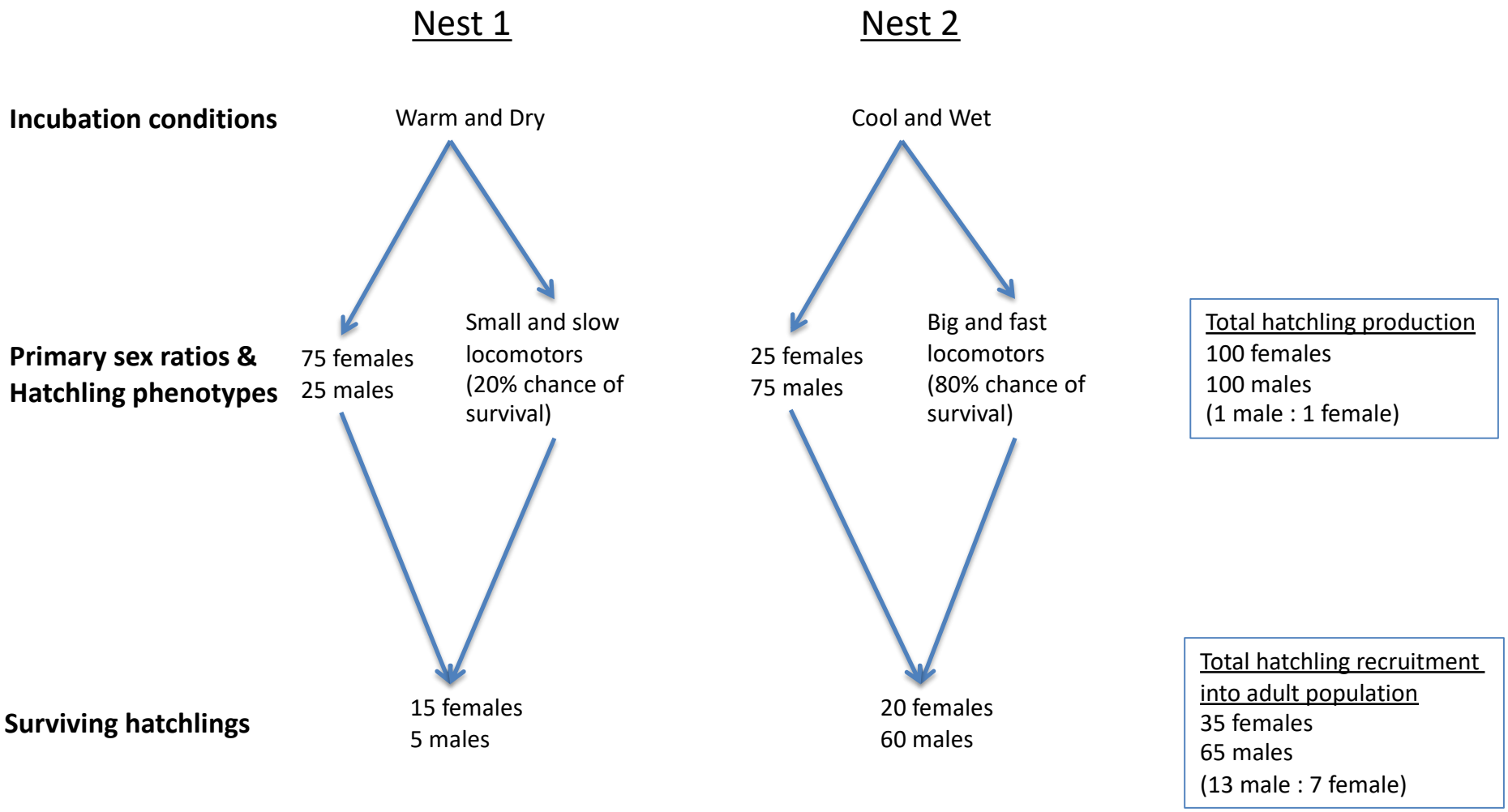

Figure 2.2: Co-variation in primary sex ratios and hatchling phenotypes with incubation conditions results in 'filtered' primary sex ratios. The sex ratios of hatchlings recruited into adult populations are altered from primary sex ratios because the conditions that produce more hatchlings of a particular sex, in this case males, also produce bigger hatchlings that are faster runners/crawlers and are likely to have lower mortality rates (Civantos & Forsman, 2000; Santidrián Tomillo *et al.*, 2014).

environmental factors such as moisture and salinity could also have negative effects on population viability (Caut *et al.*, 2010; Barrows, 2011; Bower *et al.*, 2013). Additionally, elevated mortality rates can also occur across multiple life stages as a consequence of altered incubation conditions. For instance, reduced hatchling growth rates have been linked to increased mortality rates within the first 10-18 months of life (Hare *et al.*, 2004; Dayananda *et al.*, 2016). Sub-optimal incubation conditions generally decrease hatchling quantity and quality, further reducing population viability (Hawkes *et al.*, 2007; Pike, 2014).

In summary, altered incubation conditions due to climate change may influence adult populations in four main ways: 1) altering primary sex ratios, 2) altering incubation conditions to influence hatchling phenotypes, survival and recruitment rates, 3) by giving hatchlings incubated under certain conditions long-term fitness advantages (including sex-specific survival rates) over other hatchlings, and 4) conferring reproductive advantages for females that nest in certain locations or at times that maximise hatchling quality and quantity. The degree of these changes is likely to vary due to the predicted heterogeneity of climate change and the capacity of individuals and populations to respond within necessary timeframes.

2.6 CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Research on the effects of incubation conditions on hatchling phenotypes in oviparous reptiles has largely focused on the role of temperature. The impacts of other environmental factors such as moisture, oxygen concentration and salinity have been under-investigated, although it is clear that these factors may have significant biological impacts on reptile embryonic development. Specifically, the current focus on temperature does not account for variation in other environmental factors (e.g. moisture) or the combined effects of multiple, interacting factors on hatchling phenotypes. As a result, most current predictions of reptile phenotypic responses to environmental fluctuations do not account for the full spectrum of changes that might be expected in response to climate change. In particular, Crocodilians have received little attention compared to Squamates and Testudines. Additionally, tuataras also require further attention because of their unique physiology and evolutionary history as well as their southerly habitat and subsequent adaptation to low temperatures relative to other reptiles. Future studies should also focus on species from Asia, South America and Africa rather than well-studied continents such as North and Central America, Europe and Australia. Based on the information available, expected changes to primary sex ratios will eventually lead to population-wide sex ratio imbalances, while changes to hatchling morphology and

locomotor performance will impact hatchling recruitment, possibly in a sex-dependent manner. Predicted increases in embryonic and hatchling mortality may have a greater impact on reptile adult populations than altered primary sex ratios but identifying the consequences of altered incubation conditions for adult populations is difficult. However, research on the relative effects of primary sex ratios and embryonic mortality on population viability has focused on the Testudines. The effects of climate change are likely to be spatially and temporally heterogenous, resulting in a variety of species-specific responses across the Reptilia. It is particularly important to investigate the role that moisture plays in modulating the effects of temperature on developing embryos. Increases in rainfall and sea level rise have the potential to counter the effects of warmer nesting sites and produce higher-quality offspring, and, for species with TSD, hatchlings of the less-common sex. However, high moisture levels resulting from flooding or intense rainfall may also negatively affect reptile hatching success and other phenotypic traits such as locomotor performance, potentially influencing population viability. Further studies on the role of moisture during incubation should focus on Squamates, especially considering that the diversity of the Squamata makes generalising among species difficult. Models of crocodilian and squamate population responses to altered hatchling phenotypes are particularly required. Current models of Squamate population viability focus on activity restriction in adult lizards (e.g., Kearney, 2013) and models of crocodilian population dynamics are limited. Understanding phenotypic responses to dynamic, multifaceted nesting environments is vital for conserving and managing oviparous species. To predict the impact that environmental variation will have on embryonic development, it is necessary to understand how interacting environmental factors may alter hatchling phenotypes and to incorporate this knowledge into population models. Future research should further investigate phenotypic responses to multiple environmental variables in both field and laboratory studies. Additionally, studies have not thoroughly examined the role of local substrate characteristics in influencing incubation conditions, so research is need to examine these characteristics to determine how current nesting habitat may change under predicted climatic variation. Finally, research should continue to investigate how incubation conditions ultimately shape adult populations, as well as how adults may alter their behaviour in order to optimise incubation conditions for their offspring. Reptiles are a diverse and ecologically important group of vertebrates that are particularly valuable as model species for studies on the effects of environmental variation during development. However, their diversity, especially within the *Squamata,* makes generalising among them difficult and highlights the importance of strategically directed research.

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Chapter 3. Sea turtle hatchling locomotor performance: Incubation moisture effects, ontogeny and species-specific patterns

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A flatback hatchling about to be measured.

Photo taken by Christopher Gatto.

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3.1 ABSTRACT

Incubation conditions are critical in determining numerous traits in reptilian neonates. This is particularly significant in species with low offspring survival such as sea turtle species, because of the extremely high predation rates that hatchlings face during their initial dispersal from nesting beaches. Hatchlings that develop in suboptimal nest environments are likely to be smaller, slower and more susceptible to predation than hatchlings from optimal nest environments. Previous studies have focused on the effects of temperature on hatchling traits, but few have investigated the effects of moisture concentrations, despite moisture levels in nests influencing hatchling size, sex, incubation duration, and hatching success. Here, we incubated eggs of three sea turtle species at various moisture levels and tested the terrestrial and aquatic locomotor performance of the resultant hatchlings during the frenzy and post-frenzy period. We also compared and evaluated the ontogeny of early locomotor performance for each species over the first months of life. Drier incubation conditions produced hatchlings that crawled more slowly and took longer to self-right than hatchlings from wetter incubation conditions. There was no difference in swimming performance associated with moisture treatments. We suggest that moisture in the nest environment during incubation may influence hatchling performance via their initial hydration levels. Thus, nest moisture influences terrestrial performance (i.e., escaping from the nest and dispersing across the beach), although upon entering the ocean hatchlings have opportunity to rehydrate by drinking and thus, differences in locomotor performance associated with moisture treatments cease.

3.2 INTRODUCTION

Many oviparous species lay their eggs in nests in order to reduce environmental fluctuations and optimise nest conditions (Blackburn, 1999). However, embryos can still experience considerable environmental variation in nest conditions as a result of local weather and climatic variation (Cagle *et al.*, 1993; Ackerman *et al.*, 1997). Additionally, nest location can result in considerable differences in incubation environments based on shade availability or proximity to water sources (van de Merwe *et al.*, 2006; Wood *et al.*, 2014; Hill *et al.*, 2015).

Within the vertebrates whose parental care ends with nest site selection, and hence whose eggs are exposed to the external environment, sea turtles have been the focus of numerous studies on the effects of incubation conditions on embryonic development and hatchling traits (Booth, 1998; Booth, 2006; Caut *et al.*, 2010; Lolavar & Wyneken, 2015; Booth, 2017;

Lolavar & Wyneken, 2017). Sea turtle nesting seasons can last for many months, often starting in cool, wet conditions and lasting until conditions become warm and dry (Dornfeld *et al.*, 2015). Additionally, their nesting takes place on coastal beaches, that under climate change scenarios, are predicted to be affected by increased air and sea temperatures, sea level rise, altered rainfall patterns and increased storm frequency and intensity (Fuentes *et al.*, 2010a; Fuentes *et al.*, 2010b; Pachauri *et al.*, 2014). The majority of studies on the effects of incubation conditions on sea turtles have focused on temperature. These studies showed that warmer incubation temperatures increase female hatchling production (Godley *et al.*, 2002; Godfrey & Mrosovsky, 2006) and produce smaller, weaker hatchlings (Booth, 2006; Booth, 2017) than cooler incubation temperatures.

Despite the strong effect of incubation temperature on hatchling traits, few studies have investigated the effects of other environmental factors, such as moisture. Moisture of the incubation environment has been shown to influence hatchling morphology and hatching success (Ragotzkie, 1959; Kraemer & Bell, 1980; McGehee, 1990), while more recent studies have begun to investigate how moisture influences hatchling sex ratios (Wyneken & Lolavar, 2015; Lolavar & Wyneken, 2017). In addition to potential direct effects, moisture can exert an indirect effect although alteration of other environmental factors, such as temperature (Lolavar & Wyneken, 2015). However, compared to other hatchling traits, the effect of moisture during incubation on locomotor performance of sea turtle neonates has been relatively unstudied.

Understanding the factors that determine locomotor performance in sea turtle hatchlings is important because of the importance of a brief period of extreme activity termed the 'frenzy period' (Carr & Ogren, 1960). The frenzy is characterised by heightened activity, lasting approximately 24 hours, that sea turtle hatchlings undergo as they emerge from their nest, crawl from the nest to the water and then swim rapidly and continuously to reach offshore waters as quickly as possible (Wyneken & Salmon, 1992). Hatchlings that are slower crawlers spend more time exposed to terrestrial predators and hatchlings that spend less time swimming or are slower swimmers spend more time in nearshore, predator dense zones (Whelan & Wyneken, 2007). Therefore, slower crawlers and swimmers are more likely to be preyed upon (Gyuris, 1994). Variation in hatchling performance, as a result of incubation conditions, can alter hatchling survival rates (Cavallo *et al.*, 2015). Altered hatchling recruitment may result in changed population dynamics and impacts to population viability.

The majority of hatchling dispersal occurs in the ocean and thus, hatchling swimming performance has the greatest influence on hatchling survival. Sea turtle hatchlings generally exhibit four swimming behaviours: power stroking, dog paddling, 'rearflipper kicking' and resting (Wyneken 1997). Power stroking is described by swimming with both flippers flapping in unison and generates thrust on the down stroke and occasionally on the up stroke (Booth, 2014). The dog paddling is a 'front crawl' type stroke where the hatchlings alternate protraction and retraction of diagonally opposite flippers and hind feet as they swim. This behaviour is generally used by hatchlings as they orient or breathe. Rear flipper kicking produces thrust by the hind limbs alone and is used after the frenzy. The last behaviour is resting, characterized by hatchlings flexing the flippers over the carapace and tucking the hind limbs as they passively float at the surface. This behaviour is seldom seen during the initial stages of dispersal, but hatchlings spend more time resting as they tire.

One overall measure of swimming performance is mean swim thrust, or the mean amount of thrust produced over the entire swimming trial, because this measure incorporates other attributes of swimming performance into a single value (Booth, 2009; Booth & Evans, 2011). Other attributes indicate the amount of time that hatchlings spend exhibiting certain swimming forms, such as the proportion of time spent powerstroking over an entire swimming trial, and the duration of individual powerstroking bouts. Hatchlings that spend a higher proportion of their swimming trial powerstroking or have longer powerstroking bouts are able to complete more powerstrokes and thus, are likely to produce higher mean thrust. Another attribute of swimming performance is stroke frequency during powerstroking bouts or stroke-rate during powerstroking bouts. Hatchlings that powerstroke at higher frequencies complete more powerstrokes and are likely to produce higher mean thrust. Lastly, mean maximum thrust is a measure of the maximum thrust production per powerstroke. Producing more thrust per powerstroke allows hatchlings to produce higher mean thrust. Thus, mean swim thrust provides an overall measure of swimming performance while the other attributes reflect the amount of time that hatchlings spend performing specific behaviours, the rate at which they stroke and the amount of thrust that they can produce per stoke (Booth, 2009; Booth & Evans, 2011). This allows us to directly compare hatchlings and to analyse the differences among hatchlings that result in altered swimming performance.

Here, we investigated how moisture levels during incubation influence locomotor performance by incubating eggs from three species of sea turtle in different moisture

conditions. We also evaluated the ontogeny of each species' locomotor performance and its potential consequences for population dynamics and viability. Finally, we compared the locomotor performance of the three species to identify potential differences in life history and how this may influence the impact of moisture levels during incubation. Our goal was to develop a greater understanding of how changes in moisture levels during incubation may alter hatchling recruitment and population dynamics.

3.3 METHODS

3.3.1 Egg collection

We collected eggs from Australian populations of green sea turtles (*Chelonia mydas*) from Heron Island, Queensland, flatback sea turtles (*Natator depressus*) from Curtis Island, Queensland and olive ridley sea turtles (*Lepidochelys olivacea*) from Tiwi Islands, Northern Territory. We patrolled nesting beaches at night and collected the eggs as they were laid or just after the female finished laying if we found the female covering her nest.

3.3.2 Egg transportation

Eggs were placed in plastic bags that were vacuum-sealed within 1 h of being laid following the protocol of Williamson *et al.* (2017b)**.** Vacuum sealing soon after oviposition delays the breaking of embryonic diapause by preventing eggs from being exposed to atmospheric oxygen, and ensures that embryos do not experience movement-induced mortality during transport (Rafferty *et al.*, 2013; Williamson *et al.*, 2017a). Bags of eggs were then placed in an insulated container lined with vermiculite or bubble wrap. Each container contained ice packs to maintain the temperature at 10-12°C during transport to Monash University, Melbourne, where eggs were placed into incubators filled with sand. While in transport, green sea turtle eggs were sealed for approximately 30 h; three olive ridley clutches were sealed for 72 h and the other three were sealed for approximately 24 h; one flatback clutch was sealed for 48 h and the other five were sealed for 24 h.

3.3.3 Experimental design

Each clutch of eggs was divided into three equal groups and allocated to a moisture treatment (detailed below). We collected 75 eggs from three green turtle females and 68 from a fourth female (293 eggs total). Twenty-five eggs were allocated to each moisture treatment for the first three females but for the fourth, 23 eggs were allocated to the 4% moisture treatment, 23

to the 6% moisture treatment and 22 to the 8% moisture treatment. For olive ridleys and flatbacks (180 eggs per species), we collected 30 eggs from six females of each species and allocated 10 eggs per clutch to each moisture treatment.

3.3.4 Incubation conditions

We allocated eggs from each clutch to three moisture treatments (4%, 6% and 8% w/w). These three moisture contents represent low, intermediate and high values in natural nests, while still ensuring successful embryonic development (Patino-Martinez *et al.*, 2014). All eggs were incubated at each species' pivotal temperature: 27.6°C for green turtles and 29.3°C for flatback turtles (Limpus, 2008). Olive ridley pivotal temperatures vary significantly globally (Plotkin, 2007) and are unknown for the Tiwi Island population. Thus, we maintained olive ridley eggs at the pivotal temperature of the (geographically) closest sea turtle population with measured pivotal temperatures, which in this case was 29.4°C measured for the Cape Domett flatback population (Stubbs *et al.*, 2014). Each group of eggs from every moisture treatment and clutch combination was placed in their own incubator (Hovabator 1602N, GQF Manufacturing, Georgia, USA). All incubators were housed in a temperature-controlled room set to 25°C. Eggs were buried in sand (Richgro Play Sand, 98% crystalline silica) with the top of the egg exposed so that we could monitor white spot formation as an indicator of embryonic development. Eggs that began to turn yellow, indicating embryonic death, were removed from the incubator. Once all remaining eggs had formed white spots, we covered the eggs fully with sand. As we were unable to determine whether eggs died from natural causes or from transport-induced causes, hatching success was calculated from the number of eggs that were collected. Incubator temperature was monitored daily using temperature probes (Pasco PS-2135, Roseville, California USA) buried next to the eggs.

Each incubator was filled with a known mass of dry sand and we added water to the sand to create the appropriate moisture concentration. We took multiple samples of sand (2-3g total) from around the eggs each day and calculated the moisture concentration of the sand by weighing, drying and then reweighing the sand. Moisture concentration was calculated using the following formula, where weight is measured in grams:

(1)

Using a spray bottle, we then added the amount of distilled water required to maintain the appropriate sand moisture concentration. All sand was replaced after drying to ensure total sand volume and mass did not decrease.

3.3.5 Hatchling testing

We gave hatchlings, depending on their activity levels, 24-48 h post-emergence to internalize their yolk sac before removing them from incubators. Hatchlings were marked on the carapace with non-toxic nail polish with unique patterns for identification, then we measured head width, straight carapace length (SCL), straight carapace width (SCW), flipper length (±0.01mm) using digital callipers and measured mass using electronic scales (±0.01g). We then selected 5 hatchlings at random from each incubator to be tested. Thus, 5 hatchlings were tested from each combination of moisture treatment and clutch.

Locomotor and self-righting performance testing occurred during daylight hours in a darkened room without windows and with the air temperature set at 25°C (range: 23.8-25.5°). First, we tested hatchling self-righting ability by placing hatchlings upside-down onto their carapace. Hatchlings were tested on moist, level sand and we recorded the time it took hatchlings to right themselves. Each hatchling was tested 5 times and was considered to have failed the trial if it did not right itself within 30 seconds (Rings *et al.*, 2014). We then determined the mean time it took hatchlings to self-right (failed trials were counted as 30 seconds) and the number of times a hatchling was able to successfully self-right within 30 seconds.

Next, we tested hatchling crawling ability along a level 2.4m 'racetrack' using PVC guttering lined with moist sand and a white light at one end. Hatchlings were placed at the opposite end of the racetrack to the light and were timed as they crawled towards the light. Each hatchling was tested twice to simulate a minimal crawl to the water, and we report the mean of the two trials here.

Lastly, we tested hatchling swimming ability at hatching and when the hatchlings were 4 weeks old, following the protocol of Gatto and Reina (In press). We placed hatchlings into Lycra® 'swimsuit' harnesses that did not impede their flipper movements. Each vest was attached to a load cell (PS-2201, Pasco, USA) with fishing line so that the load cell recorded the amount of thrust (Newtons) produced with each stroke taken by the hatchling. Hatchlings were encouraged to swim unidirectionally using a white light and the load cells measured

thrust production 20 times per second. Swimming performance recordings were started as soon as the hatchlings began to powerstroke. Load cells were calibrated by hanging a weight of known mass from each load cell, while water temperature was recorded using a digital aquarium thermometer. Water temperature ranged from 25.0 to 28.6°C.

Using this technique, we measured five attributes of swimming performance. First, *mean swim thrust* (N) produced over an entire swimming trial. Second, the *proportion of time that hatchlings spent power-stroking* over an entire swimming trial (%). Third, the power*stroke frequency* of hatchlings during power-stroking bouts (strokes per minute). Fourth, the *duration of power-stroking bouts* (s) and fifth, the *mean maximum thrust* (N). After hatchlings were tested during the frenzy, they were housed (conditions described below) until they were four weeks old. Swimming trials during the frenzy lasted for two h because predation rates are generally highest within the first few hours of dispersal because of higher predator densities in near-shore waters (Whelan & Wyneken, 2007). This means that swimming performance within the first few hours is likely to have a considerable effect on survival rates. Once hatchlings enter pelagic waters, predator densities decrease (Whelan & Wyneken, 2007), and so do hatchling activity levels (Wyneken & Salmon, 1992; Booth, 2009). Therefore, during post-frenzy testing when hatchlings were four weeks of age, swimming trials lasted for 30 minutes to reflect ecologically relevant periods of swimming activity. We used the same hatchlings at both 0 and 4 weeks of age but we replaced hatchlings that died at random from within the same combination of clutch and moisture treatment.

3.3.6 Hatchling housing

Hatchlings were housed in 3L and 10L plastic tanks or in glass tanks divided with plastic mesh (12.5mm grid, Aquasonic, Australia). Tanks were kept clean by a continuous flow-through system consisting of a drum filter (Faivre 60 series, Faivre, France), fluid sand bed filters (RK2 systems, USA), a protein skimmer (RK10AC, RK2 systems, USA), a UV filter (240W UV steriliser, Emperor Aquatics, USA) and an ozone steriliser (RK300MG, RK2 systems, USA). Water quality was monitored daily using OxyGuard hand-held monitors (Technolab, Australia). Water temperature was maintained between 26 and 27°C using a heater (3kW heater, Shego, Germany) and a chiller (FBT175SSD, Toyesi, Australia). Animals were maintained under a 12:12 day/night cycle and provided with UV lighting (Exo

Terra Repti Glo 5.0 25W). Turtles were fed ~2% of their body mass daily (Higgins, 2003) with commercial turtle pellets (4mm Marine float range, Ridley Aquafeed).

3.3.7 Hatchling release

After the second round of testing at 4 weeks of age, hatchlings were placed into plastic containers with holes drilled in the sides and lid and with foam lining the bottom of the containers. The hatchlings then were transported back to their natal beach and released offshore.

3.3.8 Statistical analysis

All statistical tests were performed in R (R Core Team, 2014) and our level of statistical significance was 0.05.

Differences in incubation conditions among treatment groups were tested for normality and were analysed using ANOVA and Tukey's HSD.

We used linear mixed-effects models in the lme4 package (Bates, 2007) to compare hatching success and incubation duration among moisture treatments. We used treatment as the fixed effect and clutch was the random effect.

We analysed the effect of moisture treatment on hatchling morphology using linear mixed effects models with moisture treatment as the fixed effect and clutch as the random effect.

When evaluating the effect of moisture treatment on hatchling locomotor performance, we used linear mixed-effects models with moisture treatment as the fixed effect. Our random effects were clutch and test temperature. Test temperature was the air temperature for self-righting and crawling tests and was the water temperature for swimming tests. When testing the effect of moisture treatment on the ability of hatchlings to self-right, we analysed the number of times a hatchling was able to successfully self-right as a binomial where 1 was 5 successful attempts, 0.6 was 3 successful attempts and 0 was no successful attempts.

We analysed the change in swimming performance over time using linear mixed-effects models with behavioural stage (frenzy or post-frenzy) as the fixed effect and hatchling ID, clutch, moisture treatment and water temperature as the random effects. Our hatchling ID random effect accounted for repeated measures by allowing each individual's y-intercept to vary, which accounts for differences among those individuals.

Lastly, we compared the locomotor performance among species during the frenzy and post-frenzy periods, respectively, using linear mixed-effects models. Species was the fixed effect and clutch, moisture treatment and test temperature were the random effects.

The response of each species and each measure of terrestrial locomotor performance to moisture levels during incubation was inconsistent. To determine the overall response of sea turtle terrestrial locomotor performance to moisture levels during incubation, we performed a within-study multivariate meta-analysis following the protocol of McQueen *et al.* (2017). We excluded swimming performance from the analysis because we did not observe a response to moisture treatment in any of our swimming performance indicators. We used the R package 'metafor' (Viechtbauer, 2010) and equations described in Nakagawa and Cuthill (2007) to run our weighted model with restricted maximum-likelihood to account for variation in sample sizes among tests. To account for the non-independence caused by measuring multiple locomotor performance indicators in the same hatchlings, we incorporated a variance-covariance matrix. The matrix included the within-species variance associated with each measure of terrestrial locomotor performance, and the covariances among dependent variables. The covariances were calculated using the correlation coefficients for each combination of response variables that measured the same hatchlings (i.e., between crawling speed and average time to self-right within species). To make interpretation of the results clearer, our response variables were the average time to self-right, the number of failed self-righting attempts and the average time it took hatchlings to complete crawling trials. Positive values are therefore associated with poorer locomotor performance (i.e., longer crawling times, longer self-righting times and more failed self-righting attempts). Thus, negative Zr values support the hypothesis that higher moisture levels produce faster crawlers and self-righters, while positive Zr values support the hypothesis that lower moisture levels produce faster crawlers and self-righters.

3.3.9 Animal ethics and permits

Eggs were collected under Queensland scientific purposes permit WITK17747816 (*Chelonia mydas*) and WITK18685417 (*Natator depressus*) and Northern Territory permit to take wildlife 62703 (*Lepidochelys olivacea*). Hatchlings were housed and tested under research permit 10008208 and all procedures were approved by the Monash University Biological Sciences Animal Ethics Committee (BSCI/2016/23).

3.4 RESULTS

3.4.1 Incubation conditions

The actual incubation moisture percentages in our experimental treatments (nominally 4%, 6% and 8% moisture) were statistically different within each species (Green (GR)-

t_{10}=16.569, p<0.001; Olive ridley (OR)- t_{16}=34.629, p<0.002; Flatback (FL)- t_{15}=22.872, p<0.001, Table 3.1). There was no difference in incubation temperatures among moisture treatments within any of the three species (GR- t_{10}=1.43, p=0.183; OR- t_{16}=0.919, p=0.372; FL- t_{15}=-0.385, p=0.706, Table 3.1).

Table 3.1: Mean values (± SD) for incubation conditions, incubation duration and hatching success for all three species at each treatment group.

	Species	**4%**	**6%**	**8%**	**Differences between groups**
Moisture content (% w/w)	Green	4.05 ± 0.2. n = 4	6.09 ± 0.19. n = 4	7.78 ± 0.48. n = 4	4<6<8
	Olive ridley	4.23 ± 0.25. n = 6	6.41 ± 0.2. n = 6	8.27 ± 0.08. n = 6	4<6<8
	Flatback	3.97 ± 0.31. n = 6	5.99 ± 0.3. n = 6	7.83 ± 0.24. n = 6	4<6<8
Incubation temperature (°C)	Green	27.8 ± 0.05. n = 4	27.9 ± 0.08. n = 4	27.87 ± 0.08. n = 4	4=6=8
	Olive ridley	29.29 ± 0.01. n = 6	29.28 ± 0.02. n = 6	29.31 ± 0.03. n = 6	4=6=8
	Flatback	29.46 ± 0.13. n = 6	29.41 ± 0.08. n = 6	29.43 ± 0.13. n = 6	4=6=8
Incubation duration (days)	Green	65.5 ± 3. n = 4	66.25 ± 2.22. n = 4	66.75 ± 2.87. n = 4	4=6=8
	Olive ridley	54.67 ± 0.82. n = 6	54.4 ± 0.89. n = 6	55 ± 0. n = 6	4=6=8
	Flatback	51.6 ± 1.34. n = 6	52.17 ± 0.75. n = 6	52.5 ± 1.05. n = 6	4=6=8
Hatching success (%)	Green	91 ± 6.83. n = 4	92.75 ± 3.95. n = 4	93.5 ± 5.97. n = 4	4=6=8
	Olive ridley	71.67 ± 23.17. n = 6	63.33 ± 43.2. n = 6	68.33 ± 36.56. n = 6	4=6=8
	Flatback	43.33 ± 28.75. n = 6	86.67 ± 10.33. n = 6	76.67 ± 21.6. n = 6	4<6=8

3.4.2 Hatching success and incubation duration

Moisture treatment did not influence incubation duration for green hatchlings ($F_{1,7}$=0.473, p=0.514), olive ridley hatchlings ($F_{1,9.791}$=0.782, p=0.398) or flatbacks ($F_{1,11.061}$=2.115, p=0.174). Clutch effects explained 0.88%% of the variance in incubation duration in green hatchlings, 23.97% in olive ridleys and 2.43% in flatback hatchlings.

For green (F_1=0.628, p=0.428) and olive ridley sea turtles (F_1=0.227, p=0.633), moisture treatment did not influence hatching success, but flatback eggs incubated at 4% moisture had significantly lower hatching success than eggs incubated at 6% or 8% moisture (F_1=14.713, p<0.001, Table 3.1).

3.4.3 Hatchling morphometrics

The effect of moisture during incubation on hatchling morphometrics varied with species. Moisture concentrations did not correlate with green hatchling morphometrics at any age.

In 4-week-old olive ridleys, turtles incubated at 4% moisture had narrower heads than turtles incubated at 6% or 8% moisture ($F_{1,101.88}$=12.584, p<0.001). Four-week-old olive ridleys incubated at 8% moisture were longer ($F_{1,102.14}$=10.727, p=0.001) and heavier ($F_{1,102.98}$=4.431, p=0.038) than hatchlings incubated at 4%, but neither moisture treatment differed from turtles incubated at 6% moisture. Four-week-old olive ridleys incubated at 6% moisture were wider than turtles incubated at 4% moisture ($F_{1,103.27}$=4.435, p=0.038), but neither the 6% nor 4% moisture treatments differed from the 8% moisture treatment. Lastly, 0-week-old olive ridley hatchlings incubated at 6% moisture had longer flippers than those incubated at 4% moisture ($F_{1,114.52}$=6.262, p=0.014), but hatchlings incubated at 8% moisture did not differ from the other treatment groups.

In flatbacks, 0-week-old hatchlings incubated at 8% moisture had narrower heads than those incubated at 6% or 4% moisture ($F_{1,121}$=7.866, p<0.001). At 4-weeks-old, turtles incubated at 4% moisture were heavier than those incubated at 6% or 8% moisture ($F_{1,112.85}$=4.918, p=0.029).

The statistical differences among moisture concentrations and variance explained by our random effect (clutch) can be found in Table 3.2.

Table 3.2: Mean values (± SD) for morphological variables for all species at hatching and 4 weeks. We also report the amount of variance explained by clutch effects.

	Species	Behavioural stage	4%	6%	8%	Differences between groups	Clutch (random) effects
Head width (mm)	Green	Frenzy	15.72 ± 0.57, n = 20	15.79 ± 0.55, n = 20	15.55 ± 0.61, n = 20	4=6=8	8.52%
		Post-frenzy	17.21 ± 0.62, n = 20	17.27 ± 0.55, n = 20	17.22 ± 0.41, n = 20	4=6=8	25.77%
	Olive ridley	Frenzy	14.62 ± 0.45, n = 43	14.7 ± 0.42, n = 37	14.69 ± 0.48, n = 41	4=6=8	78.14%
		Post-frenzy	15.28 ± 0.53, n = 38	15.54 ± 0.53, n = 32	15.56 ± 0.51, n = 38	4<6=8	2.4%
	Flatback	Frenzy	16.82 ± 0.33, n = 25	16.78 ± 0.29, n = 52	16.61 ± 0.37, n = 46	4=6>8	0%
		Post-frenzy	17.81 ± 0.29, n = 24	17.73 ± 0.35, n = 51	17.69 ± 0.29, n = 42	4=6=8	77.66%
SCL (mm)	Green	Frenzy	51.71 ± 2.14, n = 20	51.52 ± 2.66, n = 20	51.22 ± 1.97, n = 20	4=6=8	6.93%
		Post-frenzy	61.94 ± 3.09, n = 20	62.05 ± 2.7, n = 20	62.08 ± 2.23, n = 20	4=6=8	15.15%
	Olive ridley	Frenzy	41.38 ± 1.93, n = 43	41.47 ± 2.62, n = 37	41.63 ± 2.28, n = 41	4=6=8	63.2%
		Post-frenzy	44.53 ± 2.21, n = 38	45.29 ± 2.45, n = 32	45.25 ± 1.89, n = 38	6=8>4=6	72.84%
	Flatback	Frenzy	60.38 ± 5.18, n = 25	61.82 ± 1.72, n = 52	61.03 ± 2.48, n = 46	4=6=8	2.47%
		Post-frenzy	76.41 ± 3.17, n = 24	75.45 ± 2.25, n = 51	75.57 ± 2.14, n = 42	4=6=8	9.77%
SCW (mm)	Green	Frenzy	40.43 ± 2.1, n = 20	40.06 ± 3.26, n = 20	40.31 ± 1.88, n = 20	4=6=8	4.32%
		Post-frenzy	52.65 ± 3.64, n = 20	52.18 ± 2.82, n = 20	52.92 ± 2.63, n = 20	4=6=8	30.12%

	Olive ridley	Frenzy	34.49 ± 1.4, n = 43	34.56 ± 1.3, n = 37	34.53 ± 1.24, n = 41	4=6=8	54.3%
		Post-frenzy	39.76 ± 1.68, n = 38	40.49 ± 1.92, n = 32	40.36 ± 1.28, n = 38	8=6>4=8	53.11%
	Flatback	Frenzy	52.22 ± 2.23, n = 25	53.04 ± 1.93, n = 52	52.35 ± 2.19, n = 46	4=6=8	0.39%
		Post-frenzy	69.69 ± 2.27, n = 24	68.2 ± 2.02, n = 51	68.17 ± 2.69, n = 42	4=6=8	14.34%
Flipper length (mm)	Green	Frenzy	45.41 ± 2.44, n = 20	44.82 ± 1.8, n = 20	44.51 ± 2.29, n = 20	4=6=8	0%
		Post-frenzy	51.46 ± 2.79, n = 20	50.98 ± 1.84, n = 20	51.48 ± 1.64, n = 20	4=6=8	4.71%
	Olive ridley	Frenzy	37.44 ± 1.41, n = 43	37.49 ± 1.49, n = 37	37.81 ± 1.38, n = 41	6=8>4=6	54.21%
		Post-frenzy	39.5 ± 1.62, n = 38	39.94 ± 1.49, n = 32	39.99 ± 1.8, n = 38	4=6=8	62.99%
	Flatback	Frenzy	45.22 ± 1.64, n = 25	46.1 ± 1.3, n = 52	45.23 ± 1.85, n = 46	4=6=8	9.84%
		Post-frenzy	47.56 ± 2.02, n = 24	47.46 ± 1.52, n = 51	47.46 ± 1.16, n = 42	4=6=8	0.86%
Mass (g)	Green	Frenzy	26.18 ± 2.88, n = 20	26.36 ± 3.14, n = 20	26.38 ± 3.39, n = 20	4=6=8	17.55%
		Post-frenzy	41.98 ± 6.59, n = 20	41.25 ± 4.37, n = 20	43.08 ± 3.7, n = 20	4=6=8	26.9%
	Olive ridley	Frenzy	16.55 ± 1.52, n = 43	16.42 ± 1.71, n = 37	16.28 ± 1.87, n = 41	4=6=8	70.77%
		Post-frenzy	19.38 ± 2.86, n = 38	19.86 ± 2.55, n = 32	20.3 ± 2.71, n = 38	6=8>4=6	57.69%
	Flatback	Frenzy	40.25 ± 3.26, n = 25	39.88 ± 2.54, n = 52	39.64 ± 2.95, n = 46	4=6=8	18.59%
		Post-frenzy	65.53 ± 5.09, n = 24	62.31 ± 4.51, n = 51	61.95 ± 4.52, n = 42	4>6=8	20.9%

3.4.4 Effect of moisture on locomotor performance

Statistical results of linear mixed effects models evaluating differences in locomotor performance among moisture treatments are shown in Supplementary Table 3.1 (p252), Figure 3.1 and Table 3.3.

Moisture treatment did not influence the time it took green turtle hatchlings to self-right, how often a green hatchling was able to successfully self-right in less than 30 seconds or crawling speed (Figure 3.1, Table 3.3).

Olive ridley hatchlings incubated at 4% moisture were slower to self-right, failed to self-right more often and were slower crawlers than those incubated at 6% or 8% moisture. Hatchlings incubated at 6% were slower to self-right, failed to self-right more often and were slower crawlers than those incubated at 8% moisture (Figure 3.1, Table 3.3).

Flatback hatchlings incubated at 4% moisture were slower to self-right and failed to self-right more often than hatchlings incubated at 6% or 8% moisture. There was no difference between hatchlings incubated at 6% and 8% moisture. Moisture treatment did not influence flatback hatchling crawling speed (Figure 3.1, Table 3.3).

Moisture treatment did not affect swimming performance at hatching or at 4 weeks of age in any of the 3 species, with no difference in mean swim thrust, mean maximum thrust, powerstroke frequency, the duration of powerstroking bouts or the proportion of time spent powerstroking in hatchlings of the same species (Table 3.3).

3.4.5 Change in swimming attributes over time

Our swimming performance attributes in green and flatback hatchlings changed considerably from the frenzy to post-frenzy period, with mean swim thrust increasing in green hatchlings but decreasing in flatback hatchlings over time. This change in mean swim thrust was the same as the change in the proportion of time spent power-stroking in both species with flatback hatchlings spending less time power stroking post-frenzy, and green hatchlings, spending more time power stroking post-frenzy, compared to the frenzy. However, post-frenzy flatback hatchlings exhibited faster powerstroke frequencies and post-frenzy, green hatchlings exhibited slower powerstroke frequencies compared to frenzy hatchlings. While post-frenzy flatback hatchlings exhibited shorter powerstroke bout durations compared to the

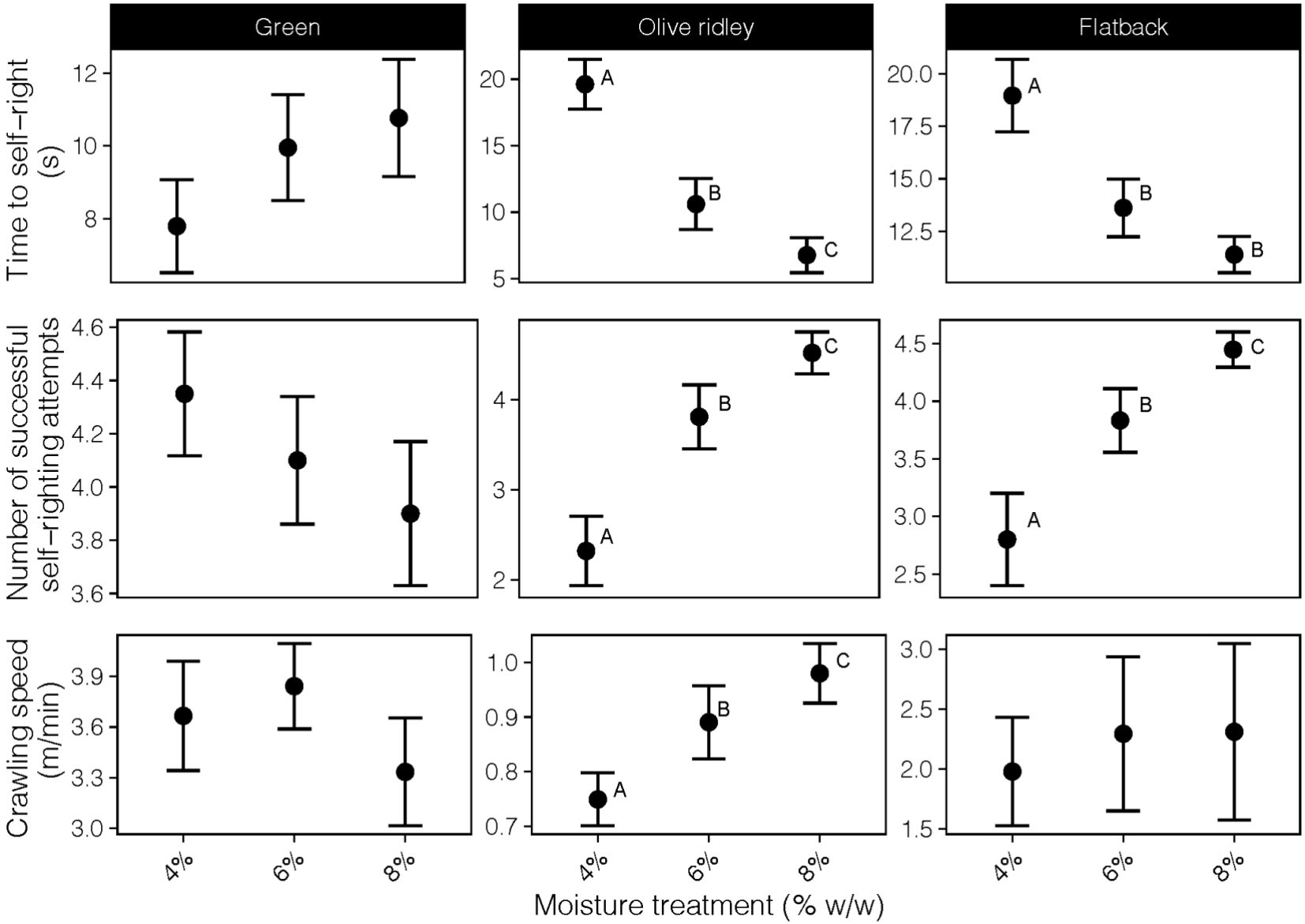

Figure 3.1: The effect of moisture treatment on our measures of hatchling terrestrial locomotor performance (mean ± standard error). Each hatchling was tested five times for self-righting ability and were tested twice on a 2.4m racetrack. Letters represent differences between moisture treatments within each species

Table 3.3: Mean values for our measures of terrestrial locomotor performance and swimming performance attributes for all three species at each treatment group and we also report the standard error. We highlight groups that differed significantly between moisture treatments in bold.

Measure of locomotor performance	Species	Behavioural stage	Moisture concentration			Differences between moisture treatments
			4%	6%	8%	
Time to self-right (s)	Green	Frenzy	7.8± 0.68, n = 20	9.95± 1.09, n = 20	10.77± 0.81, n = 20	4=6=8
	Olive ridley		**19.62± 1.23, n = 28**	**10.6± 0.59, n = 21**	**6.77± 0.57, n = 25**	**4>6>8**
	Flatback		**18.96± 1.13, n = 20**	**13.62± 0.69, n = 30**	**11.39± 0.57, n = 29**	**4>6=8**
Successful self-righting attempts (%)	Green	Frenzy	87± 0.05, n = 20	82± 0.05, n = 20	78± 0.05, n = 20	4=6=8
	Olive ridley		**46.43± 0.08, n = 28**	**76.19± 0.07, n = 21**	**90.4± 0.05, n = 25**	**4<6<8**
	Flatback		**56± 0.08, n = 20**	**76.67± 0.06, n = 30**	**88.97± 0.03, n = 29**	**4<6<8**
Crawling speed (m/min)	Green	Frenzy	3.67±0.32, n = 20	3.84±0.25, n = 20	3.33±0.32, n = 20	4=6=8
	Olive ridley		**0.75±0.05, n = 28**	**0.89±0.07, n = 21**	**0.98±0.05, n = 25**	**4<6<8**
	Flatback		1.98±0.1, n = 20	2.3±0.12, n = 30	2.31±0.14, n = 29	4=6=8
Mean swim thrust (N)	Green	Frenzy	0.0309± 0.0031, n = 20	0.0348± 0.0032, n = 20	0.0339± 0.0036, n = 20	4=6=8
		Post-frenzy	0.0548± 0.0026, n = 20	0.0538± 0.0021, n = 20	0.0523± 0.0026, n = 20	4=6=8
	Olive ridley	Frenzy	0.0109± 0.0009, n = 28	0.0099± 0.001, n = 21	0.0098± 0.0009, n = 25	4=6=8

		Post-frenzy	0.0099± 0.0012, n = 25	0.0113± 0.0014, n = 21	0.0107± 0.0011, n = 24	4=6=8
	Flatback	Frenzy	0.04± 0.0034, n = 20	0.0358± 0.0024, n = 30	0.0403± 0.0027, n = 29	4=6=8
		Post-frenzy	0.0199± 0.0035, n = 21[a]	0.0231± 0.0032, n = 30	0.0226± 0.0027, n = 28	4=6=8
Proportion of time spent power-stroking (%)	Green	Frenzy	56.11± 4.87, n = 20	55.06± 5.77, n = 20	50.29± 4.99, n = 20	4=6=8
		Post-frenzy	70.77± 2.82, n = 20	71.08± 2.72, n = 20	69.36± 3.79, n = 20	4=6=8
	Olive ridley	Frenzy	46.62± 5.29, n = 28	42.23± 5.78, n = 21	41.04± 4.96, n = 25	4=6=8
		Post-frenzy	43.49±5.43 , n = 25	52.48± 5.59, n = 21	49.44± 4.69, n = 24	4=6=8
	Flatback	Frenzy	39.46± 4.19, n = 20	37.47± 2.93, n = 30	40.29± 3.51, n = 29	4=6=8
		Post-frenzy	16.93± 4.35, n = 21 [a]	20.87± 4.26, n = 30	15.38± 3.43, n = 28	4=6=8
Stroke rate during power-stroking bouts (str/min)	Green	Frenzy	171.85± 4.99, n = 20	174.5± 4.66, n = 20	181.98± 4.65, n = 20	4=6=8
		Post-frenzy	146.36± 3.84, n = 20	150.79± 2.49, n = 20	144.38± 3.27, n = 20	4=6=8
	Olive ridley	Frenzy	183.7± 7.54, n = 28	192.08± 6, n = 21	197.33± 7.37, n = 25	4=6=8
		Post-frenzy	180.16± 8.68, n = 25	181.44± 10.99, n = 21	190.61± 8.91, n = 24	4=6=8
	Flatback	Frenzy	155.65± 2.79, n = 20	161.32± 2.63, n = 30	151.05± 2.76, n = 29	4=6=8
		Post-frenzy	255.3± 24.86, n = 21 [A]	243.02± 16.76, n = 30	229.41± 12.99, n = 28	4=6=8

Duration of power-stroking bouts (s)	Green	Frenzy	4.51± 0.46, n = 20	5.47± 0.67, n = 20	3.98± 0.44, n = 20	4=6=8
		Post-frenzy	5.48± 0.39, n = 20	5.45± 0.49, n = 20	5.07± 0.35, n = 20	4=6=8
	Olive ridley	Frenzy	4.82± 0.62, n = 28	4.49± 0.56, n = 21	3.63± 0.3, n = 25	4=6=8
		Post-frenzy	5.29± 0.56, n = 25	4.72± 0.47, n = 21	4.91± 0.49, n = 24	4=6=8
	Flatback	Frenzy	3.91± 0.51, n = 20	4.41± 0.53, n = 30	4.47± 0.48, n = 29	4=6=8
		Post-frenzy	1.78± 0.24, n = 21 [A]	2.39± 0.4, n = 30	1.98± 0.28, n = 28	4=6=8
Mean maximum thrust (N)	Green	Frenzy	0.1268± 0.0077, n = 20	0.1227± 0.0097, n = 20	0.1207± 0.0075, n = 20	4=6=8
		Post-frenzy	0.2603± 0.0103, n = 20	0.263± 0.0091, n = 20	0.2815± 0.0106, n = 20	4=6=8
	Olive ridley	Frenzy	0.0351± 0.0047, n = 28	0.041± 0.0095, n = 21	0.0345± 0.0025, n = 25	4=6=8
		Post-frenzy	0.0388± 0.0026, n = 25	0.0379± 0.0039, n = 21	0.0411± 0.0032, n = 24	4=6=8
	Flatback	Frenzy	0.2109± 0.011, n = 20	0.2218± 0.0089, n = 30	0.2272± 0.0096, n = 29	4=6=8
		Post-frenzy	0.2495± 0.0146, n = 21 [A]	0.2594± 0.1026, n = 30	0.2727± 0.0148, n = 28	4=6=8

[A] One flatback hatchling from a clutch that only produced 5 hatchlings would not crawl or swim during the frenzy. Thus, this hatchling was only measured post-frenzy when it did swim, resulting in the additional hatchling measured here.

frenzy, we did not observe a change in the duration of green hatchling power stroking bouts over time. Both flatback and green hatchlings were able to produce greater mean maximum thrust post-frenzy compared to the frenzy. Unlike green or flatback hatchlings, olive ridley hatchling swimming performance attributes did not change over time (Figure 3.2, Table 3.4).

Statistical results of linear mixed effects models evaluating change in swimming performance attributes over time are shown in Supplementary Table 3.2 (p254).

3.4.6 Difference in locomotor performance among species

There was no difference in the time it took hatchlings of different species to self-right or in the number of successful self-righting attempts.

Olive ridley hatchlings were the slowest crawlers but there was no difference between green and flatback hatchlings (Table 3.4).

During the frenzy period there was no difference between green and flatback hatchlings, although post-frenzy, green hatchlings produced higher mean swim thrust than flatback hatchlings. During both the frenzy and post-frenzy, olive ridley hatchlings produced the lowest mean swim thrust (Figure 3.2, Table 3.4).

During the frenzy, there was no difference among any of the 3 species in the proportion of time spent powerstroking, although post-frenzy, green hatchlings spent a greater proportion of time powerstroking than olive ridleys, which spent more time powerstroking than flatback hatchlings (Figure 3.2).

During the frenzy, olive ridley hatchlings had the highest powerstroke frequencies, followed by green hatchlings and lastly by flatback hatchlings. Post-frenzy, flatbacks had the highest powerstroke frequencies, followed by olive ridleys and lastly by green hatchlings (Figure 3.2).

There was no difference in powerstroking bout duration among species during the frenzy, but post-frenzy, flatbacks had the shortest powerstroke bout durations, and there was no difference between green and olive ridley hatchlings (Figure 3.2).

Flatback hatchlings produced the greatest mean maximum thrust during the frenzy, followed by green hatchlings, followed by olive ridley hatchlings. Post-frenzy, olive ridley hatchlings still produced the least mean maximum thrust, but there was no difference between green and flatback hatchlings (Figure 3.2, Table 3.4).

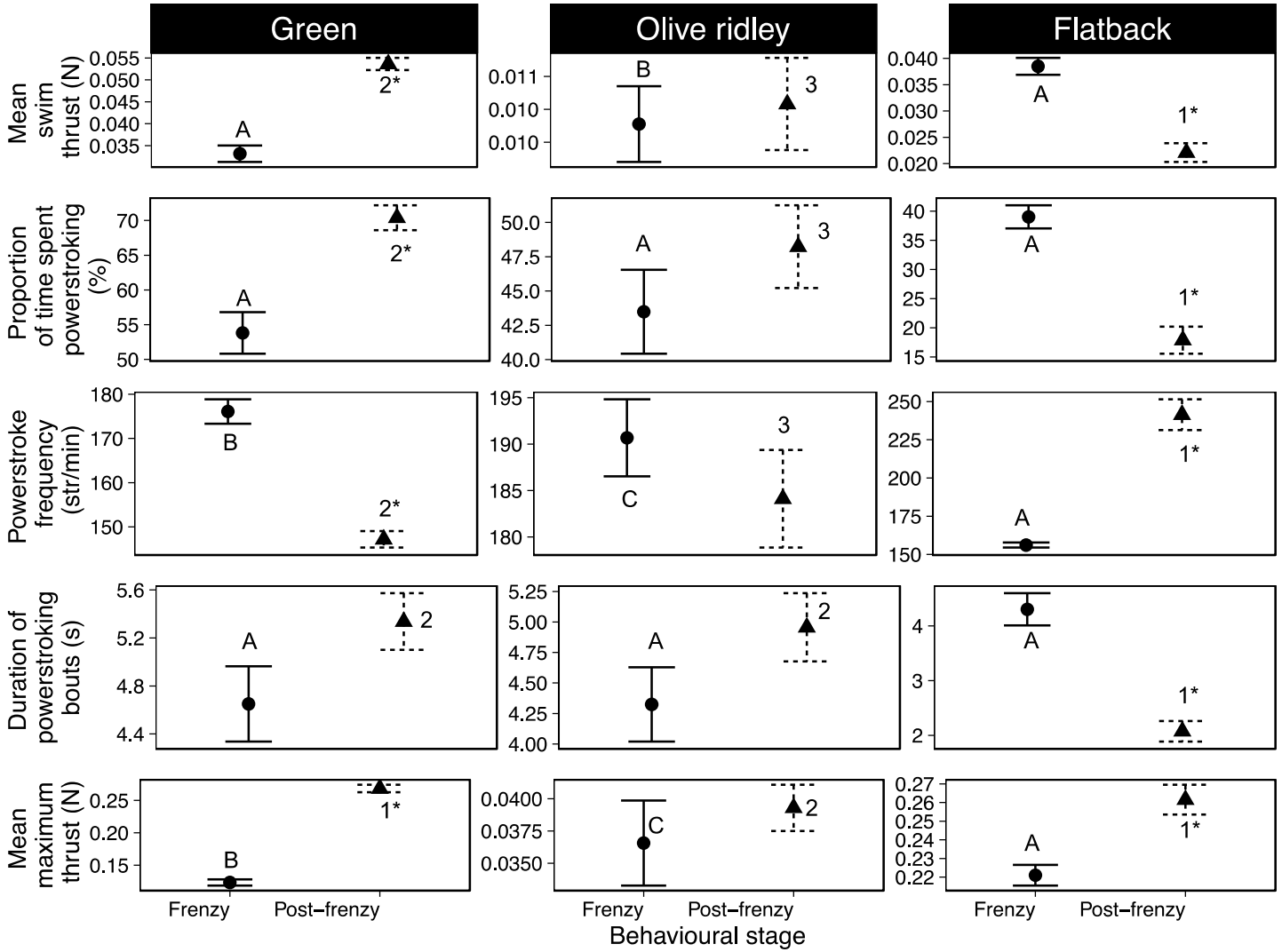

Figure 3.2 The ontogenetic change and species' comparisons of swimming performance attributes in flatback, green and olive ridley hatchlings (mean ± standard error). Asterisks represent statistical differences between frenzy and post-frenzy mean swim thrust within each species. Letters and numbers represent differences between species during the frenzy and post-frenzy, respectively. Frenzy values are presented as circles with solid lines and post-frenzy values are triangles with dashed lines

Table 3.4: Mean values for our measures of terrestrial locomotor performance and swimming performance attributes for all three species at each behavioural stage and we also report the standard error. We highlight groups with statistical differences between behavioural stages and species in bold. For differences between behavioural stages, we mark the behavioural stage where that measure of locomotor performance is higher with *.

Measure of locomotor performance	Hatchling Behaviour	Green	Olive ridley	Flatback	Differences between species
Time to self-right (s)	Frenzy	9.51± 0.84. n = 60	12.72± 1.18. n = 74	14.15± 0.82. n = 79	FL=GR=OR
Successful self-righting attempts (%)		82.33± 2.86. n = 60	69.73± 4.43. n = 74	75.95± 3.41. n = 79	FL=GR=OR
Crawling speed (m/min)		3.61± 0.17. n = 60	0.87± 0.03. n = 74	2.22± 0.07. n = 79	**OR<GR=FL**
Mean swim thrust (N)	Frenzy	**0.0332± 0.0019. n = 60**	0.0103± 0.0006. n = 74	**0.0385± 0.0016*. n = 79**	**OR<GR=FL**
	Post-frenzy	**0.0536± 0.0014*. n = 60**	0.0106± 0.0007. n = 70	**0.0221± 0.0018. n = 79**	**OR<FL<GR**
Proportion of time spent power-stroking (%)	Frenzy	**53.82± 2.98. n = 60**	43.49± 3.05. n = 74	**39.01± 1.98*. n = 79**	FL=GR=OR
	Post-frenzy	**70.4± 1.79*. n = 60**	48.23± 3.02. n = 70	**17.86± 2.32. n = 79**	**FL<OR<GR**
Powerstroke frequency (str/min)	Frenzy	**176.11± 2.76***. n = 60	190.68± 4.15. n = 74	**156.12± 1.65. n = 79**	**FL<GR<OR**
	Post-frenzy	**147.17± 1.88. n = 60**	184.11± 5.45. n = 70	**241.42± 10.19*. n = 79**	**GR<OR<FL**
Duration of power-stroking bouts (s)	Frenzy	4.65± 0.31. n = 60	4.32± 0.3. n = 74	**4.3± 0.29*. n = 79**	FL=GR=OR
	Post-frenzy	5.34± 0.24. n = 60	4.96± 0.29. n = 70	**2.07± 0.19. n = 79**	**FL<GR=OR**
Mean maximum thrust (N)	Frenzy	**0.1234± 0.0048. n = 60**	0.0366± 0.0033. n = 74	**0.221± 0.0056. n = 79**	**OR<GR<FL**
	Post-frenzy	**0.2683± 0.0058*. n = 60**	0.039± 0.0019. n = 70	**0.2615± 0.0081*. n = 79**	**OR<GR=FL**

Statistical results of linear mixed effects models evaluating differences in locomotor performance among species can be found in Supplementary Table 3.3 (p255).

3.4.7 Within study meta-analysis

Zr values that incorporate 0 indicate that moisture has no effect on that measure of terrestrial locomotor performance in that species. Thus, flatback crawling speed and all measures of green sea turtle hatchling terrestrial locomotor performance did not respond to moisture treatment during incubation. Negative Zr values indicate that wet incubation conditions produce hatchlings that are faster crawlers and are faster, more successful self-righters. Thus, higher moisture concentrations produced flatback hatchlings that were faster self-righters and also produced olive ridley hatchlings that were faster crawlers and self-righters. Overall, our within study meta-analysis confirmed that among species, hatchlings incubated at higher moisture levels were generally faster crawlers and self-righters ($\beta = -0.224$, SE = 0.092, $p<0.05$) (Figure 3.3).

3.5 DISCUSSION

3.5.1 Moisture influences terrestrial locomotion but not aquatic locomotion

Wetter incubation conditions of 6% and 8% moisture (w/w) produced flatback and olive ridley hatchlings that were able to self-right successfully more often and took less time to self-right than hatchlings incubated at 4% moisture. Olive ridley hatchlings incubated under more moist conditions (≥6% moisture) were faster crawlers than hatchlings incubated in drier conditions (4% moisture). Despite the relatively consistent influence of moisture on terrestrial locomotion as shown by our meta-analysis, moisture concentration during incubation had no effect on any of the swimming performance attributes. A potential explanation is that differences among moisture treatments can only be observed on land because sea turtle hatchlings are largely suited for aquatic locomotion where they are supported by water (Wyneken, 1996). Their different locomotion on land may reveal differences in physiology among hatchlings that aquatic locomotion does not. Alternatively, the effect of moisture on locomotion may reflect physiological effects that disappear once hatchlings enter the ocean. Sea turtle hatchlings are dehydrated when they emerge from the nest but they can recover lost water by drinking up to 12% of their body mass within the first 48 hours of entering the ocean (Reina *et al.*, 2002) and excrete excess salt through an efficient salt-secreting gland (Reina, 2000). Thus, low moisture concentrations during

incubation may have led to less hydrated hatchlings (Finkler, 1999; Hewavisenthi *et al.*, 2001) that were slower crawlers and self-righters than hatchlings from wet nests. However, once hatchlings entered the water during swimming performance testing, they could quickly rehydrate and the differences among moisture treatments disappeared (Bennett *et al.*, 1986; Reina *et al.*, 2002). Mass-specific salt gland secretion rates and concentrations are similar among sea turtle species (Reina *et al.*, 2002), suggesting that the ability of hatchlings to rehydrate is high regardless of species. Potentially, differences in hydration may also alter

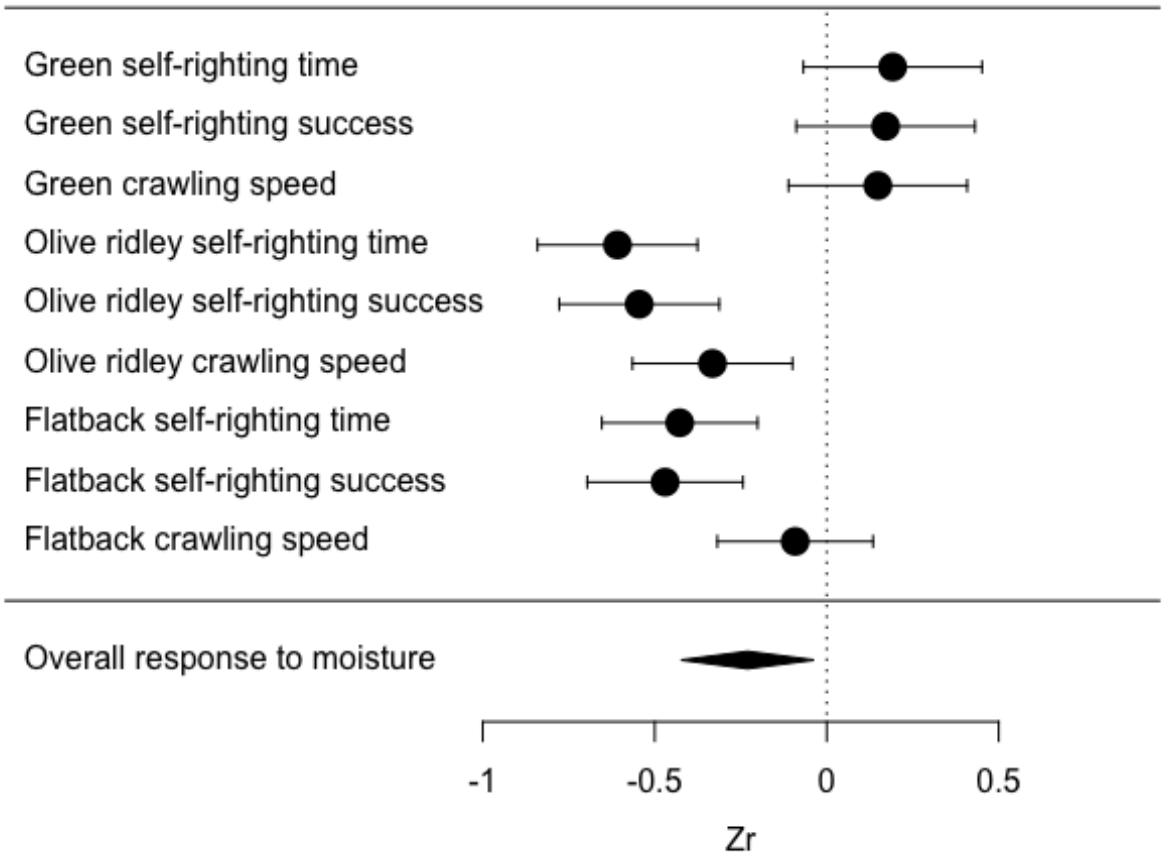

Figure 3.3 Results from our within study meta-analysis on the response of hatchling terrestrial locomotor performance to moisture levels during incubation. We report standardised effect sizes (Zr) with positive values supporting the hypothesis that lower moisture levels during incubation produce hatchlings that are faster crawlers and self-righters and negative values supporting the hypothesis that higher moisture values produce hatchlings that are faster crawlers and self-righters. Values that overlap with 0 indicate that moisture does not influence that measure of terrestrial locomotion. We present the effect sizes of each individual locomotor test and species as well as the overall effect size among species and tests

locomotor performance by influencing lactate accumulation as observed in snapping turtles (*Chelydra serpentina*) (Miller *et al.*, 1987). Additionally, previous studies in freshwater turtles have shown that differences in locomotor performance among moisture treatments remained even after hatchlings became fully hydrated, suggesting, at least in freshwater turtles, that incubation moisture concentrations may have a long-term effect on development (Miller *et al.*, 1987). It is important to consider that sea turtle hatchlings can take up to a week to emerge from the nest after 'pipping' from the egg (Rusli *et al.*, 2016) and that they slowly dehydrate within the nest during this time (Reina *et al.*, 2002). Thus, in natural nests, hatchling hydration levels change considerably from pipping to emergence and this may alter the effect of moisture during incubation on locomotor performance. Smaller olive ridley hatchlings may be more susceptible to water loss and dehydration post-emergence, compared to larger hatchlings, because of their greater surface area to volume ratio (Foley & Spotila, 1978; Hertz, 1980). While there was no statistically significant effect of moisture on green sea turtle hatchlings, our meta-analysis showed that among species, there was a significant positive correlation of moisture concentration during incubation with crawling speed and self-righting ability, at least within the range of moisture we examined.

3.5.2 Differences in ontogeny reflect life history variation

During the frenzy, all sea turtle hatchlings are benefitted by entering the ocean and escaping predator-dense nearshore waters as quickly as possible (Wyneken & Salmon, 1992), though the duration and intensity of the frenzy differs among species (Chung *et al.*, 2009b; Chung *et al.*, 2009a; Salmon *et al.*, 2009). Thus, species did not differ in the amount of time they spent power-stroking during the frenzy. However, post-frenzy flatback hatchlings showed reductions in the proportion of time spent powerstroking and the duration of powerstroking bouts. They also exhibited increased powerstroke frequencies compared to frenzied flatbacks. These behaviours may facilitate short, high intensity bursts of swimming to escape predators (Salmon *et al.*, 2009; Pereira *et al.*, 2012) in a species that has a completely neritic life history (Bolten, 2003). In comparison, post-frenzy green hatchlings spent more time powerstroking, yet had slower strokes rates during power-stroking bouts than frenzied green hatchlings. Thus, green hatchlings may maximise the proportion of time spent powerstroking post-frenzy to facilitate extended dispersals into pelagic waters (Bolten, 2003) compared to flatbacks that maximise stroke rates post-frenzy. Compared to the frenzy, flatback hatchlings experience smaller reductions in maximal metabolic rate post-frenzy than green hatchlings (Gatto et al., unpublished data). This may reflect flatback hatchlings transitioning to short, high intensity

bursts of swimming activity to escape predation in neritic waters (Salmon *et al.*, 2009; Pereira *et al.*, 2012), compared to post-frenzy green hatchlings that experience reductions in swimming intensity but remain highly active post-frenzy as they undertake extended dispersal migrations (Bolten, 2003). Though olive ridley swimming attributes did not change statistically from the frenzy to post-frenzy, changes in these attributes matched those of green turtles, potentially reflecting that their life history more closely resembles that of green turtles than flatback turtles (Bolten, 2003). Ontogenetic differences among species in their swimming performance largely appear to reflect life history variation. These life history differences lead to divergent foraging behaviours and predation pressures (Bolten, 2003; Salmon *et al.*, 2009), partially driving the variation in locomotor performance that we observed here.

3.5.3 Olive ridleys are the slowest locomotors

Among species, there was no difference in self-righting ability, although olive ridleys were slower crawlers and the slowest swimmers, as indicated by mean swim thrust compared to flatback or green hatchlings. The lower mean swim thrust of olive ridleys appears to be largely driven by their lower mean maximum thrust production, both during and post-frenzy. The considerably smaller body size of olive ridley hatchlings likely makes them less capable than larger species of producing thrust during terrestrial and aquatic locomotion, resulting in slower crawling and swimming speeds (Burgess *et al.*, 2006; Pereira *et al.*, 2012). Interestingly, olive ridley hatchlings exhibited the highest stroke rates during power-stroking bouts at emergence compared to the other species, potentially a strategy that olive ridleys use to offset their lower thrust production per stroke (Burgess *et al.*, 2006; Booth, 2009). Increases in crawling speed resulting from wetter incubation conditions may be more beneficial to olive ridley hatchlings because of their small body size and slower crawling speeds compared to other species.

3.5.4 Ecological ramifications of moisture

Although the influence of moisture during incubation on hatchling locomotor performance is limited to terrestrial locomotion, variation in moisture level on nesting beaches is likely to influence sea turtle populations. Not only are higher moisture levels, as a result of higher rainfall and sea level rise, likely to reduce nest temperatures (Lolavar & Wyneken, 2015), our data show that they will also produce hatchlings that are faster crawlers and are possibly more likely to survive initial, terrestrial phases of dispersal. Conversely, drier nests are likely

to be hotter and may produce hatchlings with reduced terrestrial locomotor ability. However, the impact of moisture variation will not influence species equally. Green sea turtles appear to be considerably less sensitive to moisture levels during incubation than either flatback or olive ridley hatchlings, potentially reflecting their generally greater tolerance of extreme temperatures compared to other species (Howard *et al.*, 2014). The greater sensitivity to moisture of olive ridley hatchlings compared to other species may result from their smaller egg size and thus, greater egg surface area to volume ratio (Ackerman *et al.*, 1985). However, the role of egg size on the sensitivity of developing sea turtle embryos to moisture requires further investigation, particularly considering that the intermediate sized eggs of green turtles were less response to moisture during incubation than the large eggs of flatback turtles. Eggshell structure is similar among sea turtle species and is unlikely to contribute to species' sensitivity to moisture (Phillott & Parmenter, 2006). Within species, populations are likely to experience significantly different changes in moisture levels because changes in precipitation will vary regionally (Pachauri *et al.*, 2014). Thus, populations that experience an increase in moisture may experience greater hatchling survival during the crawl from nest to ocean and those in drier areas may experience decreases in hatchling survival. Within populations, moisture concentrations and thus, hatchling terrestrial locomotor performance, will vary both temporally throughout the nesting season and spatially depending on proximity to the ocean and to vegetation (Wood *et al.*, 2000; Dornfeld *et al.*, 2015). Overall, sea turtle population responses to moisture will vary among species, populations, beach characteristics and even among nest locations. Differences in beach characteristics and nest location can result in variation in substrate grain size (Karavas *et al.*, 2005; Chen *et al.*, 2007), vegetation type and density (Hays *et al.*, 1995) and can alter the elevation of the nest relative to the ocean (Wood *et al.*, 2000), all of which influence the amount of moisture in the nest and can influence the availability of moisture to developing embryos (Kraemer & Bell, 1980; Bouchard & Bjorndal, 2000; Foley *et al.*, 2006). Sea turtles have been shown to shift their nesting phenology and nest-site selection in response to altered air and sea temperatures (Mazaris *et al.*, 2013; Lamont & Fujisaki, 2014). Whether nesting females will do the same in response to moisture or indeed whether they are capable of detecting these differences remains to be seen. However, sand moisture concentrations can rapidly vary, both spatially with depth and temporally in response to rainfall, making moisture an unreliable cue for nesting females (Wood *et al.*, 2000). Females that do shift their nest sites are likely to experience fitness advantages as a result of increased hatchling survival during dispersal (Lamont & Fujisaki, 2014).

3.5.5 Conclusions

In conclusion, wetter incubation conditions produce sea turtle hatchlings that crawl faster, take less time to right themselves when over-turned, and are able to successfully right themselves more often than hatchlings from dry incubation conditions. Green hatchlings were the least sensitive to moisture and did not respond to incubation moisture concentrations in any of our performance tests. None of the three species we tested varied in their swimming performance in response to moisture concentrations. Flatbacks were the largest hatchlings and thus, required more water to be normally hydrated. In comparison, olive ridleys were the smallest hatchlings and could dehydrate more quickly in air compared to other, larger species. Differences in hydration potentially influence terrestrial locomotion, but these differences disappear once hatchlings enter the ocean and likely rehydrate. Future studies on the effects of moisture during incubation should focus on pinpointing the mechanisms behind the effect of moisture on crawling speeds, and consider incubating eggs at higher moisture levels that may highlight differences among hatchlings and reflect potential incubation conditions under climate change scenarios. Research should also investigate multiple, interacting environmental variables, such as temperature and moisture, that more realistically reflect natural nests. When comparing species, the divergent behaviours of all three species we examined largely reflected differences in life history.

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Chapter 4. The role of incubation environment in determining sea turtle hatchling thermal tolerance

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Emerging green hatchlings from the Lang Tengah Turtle Watch hatchery.

Photo taken by Christopher Gatto.

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4.1 ABSTRACT

Warming global temperatures are predicted to reduce population viability in many oviparous ectothermic taxa, with increased embryonic mortality likely to be one of the main causes. While research on embryonic upper thermal limits is extensive, hatchling thermal tolerance has received less attention and our understanding of how incubation conditions influence hatchling thermal tolerance is limited. Here, we report hatchling hydration and thermal tolerance following incubation in dry and wet conditions. We used packed cell volume and total protein as indicators of hydration and measured the Critical Thermal Maximum (CT_{max}) of hatchlings in air. Neither hatchling hydration nor thermal tolerance were influenced by moisture during incubation. However, hatchlings from moister nests had longer incubation durations (wet: 60.11 vs. dry: 54.86 days) and using incubation duration as a proxy for incubation temperature, hatchlings from cooler nests had significantly lower CT_{max} (wet: 39.84°C vs. dry: 40.51°C). Thus, warmer conditions resulted in higher thermal tolerance in hatchlings. Neonates of ectothermic species may have greater plasticity in their thermal tolerance than previously thought, but their ability to adapt to increasing temperature is likely to be limited. Additionally, common management techniques, such as watering and shading nests, may only reduce embryonic mortality at the cost of decreased hatchling thermal tolerance, potentially resulting in increased hatchling mortality during emergence as hatchlings crawl to the ocean. Thus, nesting-site management interventions designed to reduce embryonic mortality will need to consider mitigation of the possible effects of those interventions on hatchling mortality.

4.2 INTRODUCTION

Environmental factors, such as temperature, moisture, oxygen concentration and salinity, all influence multiple traits and phenotypes in a variety of taxa (Alberts *et al.*, 1997; Booth, 2006; Owerkowicz *et al.*, 2009; Caut *et al.*, 2010; Bower *et al.*, 2013). These effects can be long lasting (Elphick & Shine, 1998; Freedberg *et al.*, 2004), and when environmental conditions affect large enough areas of a species' nesting habitat, can significantly affect species at a population level (Hawkes *et al.*, 2007; Santidrián Tomillo *et al.*, 2012).

Research into the effects of nest conditions has been extensive in reptiles, particularly sea turtles. Sea turtles provide no parental care and nest over many months. Thus, developing embryos experience considerable variation in incubation conditions as the year progresses, in

addition to spatial variation in the microclimate within the nest (Wallace *et al.*, 2004; Ralph *et al.*, 2005). This variation has important implications for hatchling survival (Burgess *et al.*, 2006; Cavallo *et al.*, 2015). Most studies have investigated temperature effects, showing that warmer nests produce higher proportions of females (Mrosovsky, 1994; Godley *et al.*, 2002; Godfrey & Mrosovsky, 2006) and smaller, weaker sea turtle hatchlings (Burgess *et al.*, 2006; Fisher *et al.*, 2014; Booth, 2017). These smaller hatchlings are less capable of escaping wave zones, are at higher risk of predation and therefore are likely to have higher rates of mortality than larger, stronger hatchlings (Booth & Evans, 2011; Cavallo *et al.*, 2015), potentially leading to reduced survival of female hatchlings and more balanced sex ratios than previously thought. However, persistent production of female-biased primary sex ratios eventually leading to female-biased adult populations, has been thought to be the greatest threat to sea turtle population viability (Hawkes *et al.*, 2007; Fuentes *et al.*, 2010; Kallimanis, 2010).

However, recent research suggests that the largest threat to sea turtle populations may be embryonic mortality as a result of increased nest temperatures (Laloë *et al.*, 2014; Santidrián Tomillo *et al.*, 2014; Santidrián Tomillo *et al.*, 2015). Both laboratory and in-situ studies have shown that sea turtle embryonic mortality increases significantly at temperatures above 34°C (Valverde *et al.*, 2010; Maulany *et al.*, 2012; Howard *et al.*, 2014), although some laboratory studies have observed 0% hatching success at temperatures as low as 32°C in leatherback and loggerhead turtles (Binckley *et al.*, 1998; Fisher *et al.*, 2014). With sand temperatures regularly exceeding 34°C on many nesting beaches including in Australia, Central America and Asia (Matsuzawa *et al.*, 2002; Valverde *et al.*, 2010; Sim *et al.*, 2015), reduced hatchling production is expected to be a major cause of sea turtle population decline (Santidrián Tomillo *et al.*, 2012). However, the impact of climate change on hatchling recruitment may extend beyond the nest, because high sand temperatures also increase hatchling mortality when dispersing hatchlings overheat as they crawl from nest to ocean. Temperature-driven hatchling mortality events observed in Australia, the USA and Costa Rica are becoming increasingly common and are likely to exacerbate the effects of embryonic mortality within nests (Santidrián Tomillo *et al.*, 2012; Foley, 2017; Lodge, 2017).

While considerable effort is being made to maximise hatching success on nesting beaches by relocating eggs and increasing shade (Garcıía *et al.*, 2003; Fuentes *et al.*, 2011; Fuentes *et al.*, 2012; Hill *et al.*, 2015), our understanding of how to increase hatchling survival from the nest

to ocean is limited. This includes understanding which factors influence the thermal tolerance of dispersing hatchlings. Considering the importance of the incubation environment for sea turtle hatchlings (Godfrey & Mrosovsky, 2006; Booth, 2017), it is possible that hatchlings incubated under different conditions may have varying tolerances to extreme temperatures. Here, we investigate the role of incubation moisture concentrations in determining sea turtle hatchling thermal tolerance. We also examine hatchling hydration as the potential mechanism behind any response of thermal tolerance to nest moisture. Hydration has been shown to influence the thermal tolerance of reptiles (Plummer *et al.*, 2003), with more hydrated individuals being able to tolerate warmer temperatures. We measured hatchling hydration at emergence using packed cell volume and total protein as indicators and then tested the critical thermal maximum (CT_{max}) of the same hatchlings. The CT_{max} is the temperature at which the hatchling cannot remove itself from conditions that would lead to death due to locomotor impairment (Lutterschmidt & Hutchison, 1997; Drake & Spotila, 2002). It is an indicator of an individual's thermal tolerance without negative long-term effects. Temperature-driven hatchling mortality, like embryonic mortality, is becoming more frequent and an emerging threat to sea turtle population viability. This study is an initial investigation into the factors that determine hatchling thermal tolerance and highlights potential management strategies to minimise temperature-driven hatchling mortality events on increasingly warming nesting beaches (Fuentes *et al.*, 2010; Laloë *et al.*, 2014).

4.3 METHODS

4.3.1 Study sites, dates and species

This study was conducted at the Lang Tengah Turtle Watch hatchery on Kuala Abang beach, Dungun, Terengganu, Malaysia. Entire clutches of eggs (N=20 clutches) were collected from nesting green turtle females (*Chelonia mydas*) on Kijal beach, 42km south of the hatchery, from the 6-15 May, 2018 (1_{st} collection) and another twenty entire clutches of eggs were collected from the 1-9 June, 2018 (2_{nd} collection).

4.3.2 Egg collection and transport

Each clutch was collected in a bucket during oviposition, covered in sand, transported to the hatchery and buried within 6 hours. Nest chambers were dug in the centre of a $1m_2$ plot within the hatchery, to a depth of 70cm. Plots were arranged in 3×8 grid with wet nests on

one side of the grid and dry nests on the other. Wet and dry nests were separated by at least one empty plot.

For each clutch of eggs, every third egg was weighed before being placed in the egg chamber. We placed a Thermochron ibutton (Temp-log Australia, DS1921G#F50) in the centre of five wet nests and five dry nests to record nest temperature every three hours throughout incubation.

4.3.3 Nest moisture content

Prior to collecting the first clutch of eggs, we ran a pilot study to establish an appropriate watering regime to maintain our wet nests at 8% v/v moisture and our dry nests at 4% v/v moisture. Moisture content (% v/v) was determined using a soil moisture probe (Pasco ECH$_2$O EC-5) at depths of 35cm, 50cm and 70cm. The probe was calibrated using a calibration curve created with sand from the hatchery of known moisture content. To create sand of known moisture content, we collected sand from the hatchery and dried it until the mass of the sand stopped decreasing (i.e. all water in the sample had evaporated). We then measured a known volume of the dry sand and added a known volume of water to produce sand of different moisture concentrations.

Once nests were placed in the hatchery, we measured the moisture content of each plot daily and added the necessary volume of water to maintain the predetermined moisture content. In dry nests, sand moisture content naturally stayed above 4%, so no water was added to these nests. All hatchlings used in this study were from the 2$_{nd}$ collection (nests S21-S40), although we included some nest moisture and temperature data from the 1$_{st}$ collection (nests S1-S20).

4.3.4 Hatchling morphology

Upon emergence, we selected five hatchlings at random from each nest and measured their straight carapace length (SCL) (±0.01mm) and straight carapace width (SCW) (±0.01mm) using digital callipers (Economy 150mm), as well as mass (±0.5g) using electronic scales (BM series H-3000). Hatchlings were collected as soon as they emerged and were measured within 30 minutes of collection. On average, hatchling measurements, hydration measurements and thermal tolerance testing was completed within 120 min of hatchling collection. Any hatchlings not chosen for testing were released immediately or after sunset for hatchlings that emerged during daylight.

4.3.5 Hatchling hydration

To measure hatchling hydration, we took a 100 μL sample of blood from the dorsal external jugular vein at the back of the neck using a 25G needle (Neolus) and 1mL syringe (Terumo) within 60 min of emergence from the nest. Samples were transferred to heparinised capillary tubes (Livingstone) and centrifuged at 11,000 rpm for 4 min (LW Scientific Zipocrit centrifuge). These samples were used to calculate % packed cell volume (PCV) and total protein ± 2 g/L (TP), which was measured from the plasma with a standard refractometer (RHCN-200ATC, NISupply, CA, USA).

4.3.6 Hatchling thermal tolerance

After blood sampling, we tested each hatchling's critical thermal maximum (CT_{max}) using a modification of the technique of Drake and Spotila (2002). First, we measured initial body temperature using a thin, fast response temperature probe (PASCO PS-2135) inserted a few millimetres into the cloaca. We then placed the hatchling into a bucket lined with a 2cm layer of sand and a second temperature probe taped to the bottom of the bucket underneath the sand. Temperature probes were read using a PASCO PASport Xplorer (PS-2000) and PASport Quad temperature sensor (Pasco model PS-2143).

We then placed a heat lamp (Exo Terra, Infrared 150W) 20cm above the surface of the sand which heated the sand at approximately 1°C/min. During this time, the hatchling was allowed to freely crawl around the bucket. We continuously observed the hatchling until it began to display 'uncoordinated' movements, at which point we recorded sand temperature. Uncoordinated movements are characterised by sporadic bouts of carapace rubbing with the front flippers, wiggling from side to side and jerky movements (Drake & Spotila, 2002). We further heated the hatchling until it began to display 'uncontrolled' movements. Uncontrolled movements are characterised by continuous flapping of the front flippers and a general stiffening of the hatchling such that it is unable to crawl (Lutterschmidt & Hutchison, 1997; Drake & Spotila, 2002). When a hatchling displayed these behaviours, we immediately removed it from the bucket, measured its body temperature, designated as its CT_{max}, recorded sand temperature and recorded the elapsed time.

Once we recorded the hatchling's CT_{max}, we placed it in a container of ambient seawater, where it was monitored continuously until we observed normal swimming behaviours (usually within 30-60 s). All hatchlings recovered and swam normally.

We subsequently held the hatchlings in a bucket lined with sand and covered with a damp cloth until all trials were completed. We then released the hatchlings at the ocean's edge after sunset.

4.3.7 Data analysis

We compared incubation duration and moisture levels between wet and dry treatments using a Student's t-test.

The effect of incubation conditions on morphology, hatchling hydration and thermal tolerance were evaluated using linear mixed effects models with nest moisture content (wet or dry) as the fixed effect and nest ID as the random effect in order to account for maternal effects such as egg mass and unknown differences among nests during incubation. Any remaining variation can be attributed to the incubation conditions.

Relationships among hatchling hydration, thermal tolerance, morphological measurements and incubation duration were also analysed using linear mixed models using nest ID as the random effect.

For nests where we were able to collect temperature data, we used linear models to investigate the relationship between mean nest temperature and incubation duration.

Models were run in R (R Core Team, 2014) using the lme4 package (Bates, 2007). Our level of significance was 0.05 and p-values were generated using the lmerTest package (Kuznetsova *et al.*, 2017).

All models were tested for independence, normality and homogeneity of variance.

4.3.8 Animal ethics and permits

All experimental procedures were approved the by the Monash University Biological Sciences Animal Ethics Committee (approval BSCI/2018/08) and Terengganu State Fisheries Office (reference SEATRU/RES/17/01).

4.4 RESULTS

4.4.1 Nest moisture content, incubation duration and hatchling morphology

Mean values for all measurements can be found in Table 4.1.

Mean sand moisture content in dry nests was approximately, although significantly, 3% v/v lower than those in wet nests ($t_{17.985}$=24.978, $p<0.001$). Hatchlings incubated in wet conditions took approximately 6 days longer to hatch than hatchlings incubated in dry conditions ($t_{17.998}$=6.414, $p<0.001$). There was no difference in mass ($F_{1,17.735}$=1.187,

p=0.291), SCL ($F_{1,18.184}$=0.364, p=0.554) or SCW ($F_{1, 18.208}$=0.331, p=0.572) between hatchlings incubated in wet vs. dry conditions. Nest ID (random effect) explained 84.52%, 57.31% and 43.56% of the variation in mass, SCL and SCW, respectively.

Table 4.1: Effects of moisture treatment on nest environment, hatchling morphometrics, hydration and thermal tolerance. Values are reported as mean ± SD with their respective units. Statistically significant differences between wet and dry incubated hatchlings are in bold.

Measurement	Dry incubation	Wet incubation	Comparison between wet and dry nests
Nest moisture content	**4.98 ± 0.24 % v/v**	**7.89 ± 0.23 % v/v**	**$t_{17.985}$=24.978, p<0.001**
Incubation duration	**54.86 ± 1.87 days**	**60.11 ± 1.63 days**	**$t_{17.998}$=6.414, p<0.001**
SCL	46.45 ± 1.92 mm	46.09 ± 1.75 mm	$F_{1,18.184}$=0.364, p=0.554
SCW	36.05 ± 1.74 mm	36.33 ± 1.3 mm	$F_{1, 18.208}$=0.331, p=0.572
Mass	20.97 ± 2.27 g	22.17 ± 1.71 g	$F_{1,17.735}$=1.187, p=0.291
Packed cell volume	32.57 ± 4.53 %	30.47 ± 4.72 %	$F_{1,17.556}$=2.257, p=0.151
Total protein	54.15 ± 5.09 g/L	53.91 ± 5.31 g/L	$F_{1,18.222}$>0.001, p=0.976
Initial body temperature	29.04 ± 1.32 °C	28.73 ± 1.22 °C	$F_{1,17.951}$=0.16, p=0.694
Critical thermal maximum	40.51 ± 1.09 °C	39.84 ± 1.14 °C	$F_{1,17.812}$=4.371, p=0.051
Initial sand temperature	29.4 ± 1.76 °C	29.06 ± 1.39 °C	$F_{1,17.886}$=0.414, p=0.528
Sand temperature at onset of uncoordinated movements	33.68 ± 1.82 °C	32.38 ± 2.11 °C	$F_{1,16.4}$=2.05, p=0.171
Final sand temperature	37.09 ± 2.07 °C	37.28 ± 1.74 °C	$F_{1,17.705}$=0.201, p=0.66

4.4.2 Hatchling hydration

There was no difference in packed cell volume (PCV) ($F_{1,17.556}$=2.257, p=0.151, Table 4.1) or total protein (TP) ($F_{1,18.222}$<0.001, p=0.976, Table 4.1) between hatchlings incubated in wet or dry conditions. Nest ID explained 30.48% of the variation in PCV and 49.38% of the variation in total protein.

Hatchlings with higher PCV also had higher total protein ($F_{1,85.359}$=8.012, p=0.006, R^2=0.021), although the relationship was weak. Nest ID explained 40.76% of the variance.

4.4.3 Hatchling thermal tolerance

There was no difference in CT_{max} between hatchlings incubated in wet (40.51 ± 1.09°C) and dry conditions (39.84 ± 1.14°C) ($F_{1,17.812}$=4.371, p=0.051, Table 4.1). Nest ID explained 21.67% of the variation. There was no difference in the initial body temperature ($F_{1,17.951}$=0.16, p=0.694, Table 4.1) or initial sand temperature during thermal tolerance testing ($F_{1,17.886}$=0.414, p=0.528, Table 4.1) of hatchlings incubated in either wet or dry conditions. Nest ID explained 79.99% of the variation in initial body temperature. Additionally, there was no difference in the final sand temperature (i.e., the sand temperature at which CT_{max} was reached) between wet and dry incubated hatchlings ($F_{1,17.705}$=0.201, p=0.66, Table 4.1).

4.4.4 Relationships among hatchling hydration, initial body temperature and thermal tolerance

When evaluating hatchlings, irrespective of their incubation conditions, a hatchling's initial body temperature did not influence their CT_{max} ($F_{1,79.95}$=0.566, p=0.454**,** R^2=0.033) or the time that hatchlings took to reach their CT_{max} ($F_{1,87.35}$=0.153, p=0.697, R^2=0.105). Packed cell volume did not influence hatchling CT_{max} ($F_{1,85.978}$=0.028, p=0.895, R^2=-0.006). Hatchlings with higher total protein values had lower CT_{max} ($F_{1,79.99}$=4.569, p=0.036, R^2=0.06), although this relationship was weak. Nest ID explained 31.36% and 25.15% of the variation, respectively. There was no relationship between CT_{max} and hatching success ($F_{1,32.11}$=0.83, p=0.37, R^2=0.02).

4.4.5 Effect of body size on hatchling thermal tolerance and hydration

Longer hatchlings had a higher CT_{max} ($F_{1,80.151}$=9.0284, p=0.004, R^2=0.057), although the relationship was weak, and hatchling mass ($F_{1,35.952}$=3.7258, p=0.061, R^2=0.015) and SCW

($F_{1,89.487}$=0.437, p=0.51, R^2=-0.006) did not influence CT_{max}. Nest ID explained 31.38%, 31.26% and 27.18% of the variation in CT_{max} with SCL, mass and SCW, respectively. Although SCL ($F_{1,87.727}$=3.17, p=0.078, R^2=0.244) and SCW ($F_{1,82.842}$=0.005, p=0.943, R^2=0.037) did not influence hatchling initial body temperature, mass did, with heavier hatchlings having lower initial body temperatures ($F_{1,82.623}$=5.931, p=0.017, R^2=0.31). Nest ID explained 76.3% (SCL), 79.13% (SCW) and 72.36% (mass) of the variation.

4.4.6 Nest temperature, incubation duration and thermal tolerance

We used incubation duration as a proxy for incubation temperature. Hatchlings that had shorter incubation durations and thus, would have incubated at higher temperatures (Van Damme *et al.*, 1992; Matsuzawa *et al.*, 2002) had significantly higher CT_{max} compared to hatchlings with longer incubation durations (i.e. lower incubation temperatures) ($F_{1,19.564}$=6.372, p=0.02, R^2=0.105) (Figure 4.1). Nest ID explained 18.04% of the variance.

To estimate the incubation temperatures experienced by the hatchlings that we tested, we plotted the relationship between incubation duration and incubation temperature using all of the nests (in both collection rounds) for which we were able to record incubation temperatures. Over both collection rounds, we recorded incubation temperatures for 12 nests (Table 4.2) and found a significant negative linear relationship between incubation duration and mean incubation temperature (t_{10}=-2.409, p=0.037, R^2=0.304) (Figure 4.2). Extrapolating from this model using the incubation durations of all hatchlings that were tested for thermal tolerance, we predict that nest temperatures would have ranged from 28°C to 31°C. Of the two nests in the 2nd collection that we did record incubation temperatures for, nest S21 (wet conditions) had a mean incubation temperature of 28.5°C and an incubation duration of 59 days. Nest S26 (dry conditions) had a mean incubation temperature of 30.45°C and an incubation duration of 53 days.

4.5 DISCUSSION

The aim of this study was to measure the response of sea turtle hatchling hydration and thermal tolerance to moisture concentrations during incubation. Moisture concentrations during incubation did not influence hatchling hydration levels. The structure, thickness and water permeability of reptile eggs varies considerably, with sea turtle eggs generally considered to lay 'pliable' eggshells with intermediate water permeability compared to other reptiles (Packard & Packard, 1980; Kusuda *et al.*, 2013). It is possible that in our study, we

Table 4.2: Mean nest temperatures and incubation durations for all nests that contained temperature probes. We included nests from a concurrent study that also manipulated moisture levels, but were not included in this study (1st collection), and the two nests that successfully recorded nest temperature from this study (2nd collection). Means are given as ± standard deviation.

Collection round	Nest	Moisture level	Moisture concentration (% v/v)	Mean nest temperature (degrees C)	Incubation duration (days)
1st collection	S3	Wet	7.96 ± 1.35	29.57 ± 0.78	59
	S7		7.24 ± 1.25	28.84 ± 0.72	56
	S11		8.16 ± 1.1	28.9 ± 0.74	61
	S15		8.28 ± 1.11	29.33 ± 0.85	55
	S19		7.72 ± 1.45	29.31 ± 0.61	60
	Mean		**7.87 ± 0.41**	**29.19 ± 0.31**	**58.2 ± 2.6**
	S4	Dry	4.74 ± 1.06	29.99 ± 0.8	53
	S8		4.81 ± 0.88	29.41 ± 0.55	55
	S12		4.7 ± 0.87	29.75 ± 0.92	54
	S16		4.85 ± 0.65	29.92 ± 0.68	57
	S20		4.61 ± 0.68	29.47 ± 0.84	53
	Mean		**4.74 ± 0.09**	**29.71 ± 0.26**	**54.4 ± 1.7**
2nd collection	S21	Wet	7.77 ± 0.92	28.5 ± 0.78	59
	S26	Dry	4.93 ± 0.8	30.45 ± 0.96	53
Total	**Mean**	**Wet nests**	**7.86 ± 0.37**	**29.08 ± 0.4**	**58.3 ± 2.3**
		Dry nests	**4.77 ± 0.11**	**29.83 ± 0.38**	**54.2 ± 1.6**

observed no response of hatchling hydration and thermal tolerance to moisture treatment because our treatments did not induce a large enough change in egg water content. This may result from the eggshells altering their permeability to water depending on their hydration state (Lutz *et al.*, 1980; Lillywhite & Ackerman, 1984) or potentially because our eggs contained enough water to survive our chosen treatments (Hewavisenthi *et al.*, 2001). Additionally, moisture concentration did not directly influence thermal tolerance. Instead, we found that moisture levels altered incubation temperatures, which in turn modified hatchling thermal tolerance. We conclude that considering multiple environmental factors when assessing the role of incubation conditions in determining hatchlings traits is vital. As a result of some of our temperature probes malfunctioning, we used incubation duration as a proxy for incubation temperature because of the strong and reliable relationship between them (Van Damme *et al.*, 1992; Matsuzawa *et al.*, 2002). For example, loggerhead turtle incubation

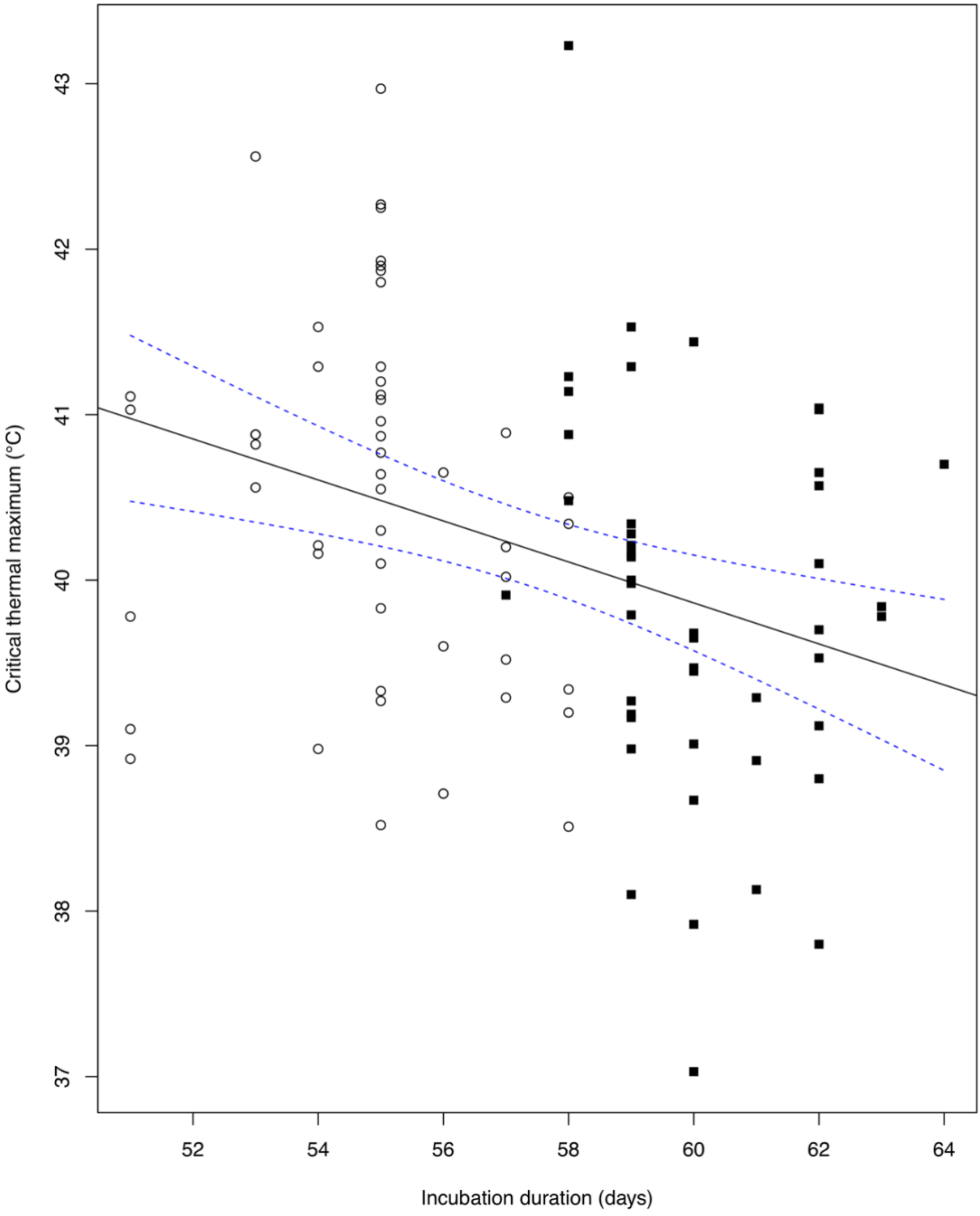

Figure 4.1: The relationship between incubation duration and hatchling critical thermal maximum. The dashed blue lines represent the 95% confidence intervals, filled black squares represent wet nests and unfilled circles represent dry nests. Hatchlings with short incubation durations are likely to have incubated at warmer temperatures than hatchlings with longer incubation durations. The relationship between incubation duration and hatchling critical thermal maximum is described by the equation, Critical Thermal Maximum (°C) = 47.3 – 0.12 × d, where d = incubation duration (days)

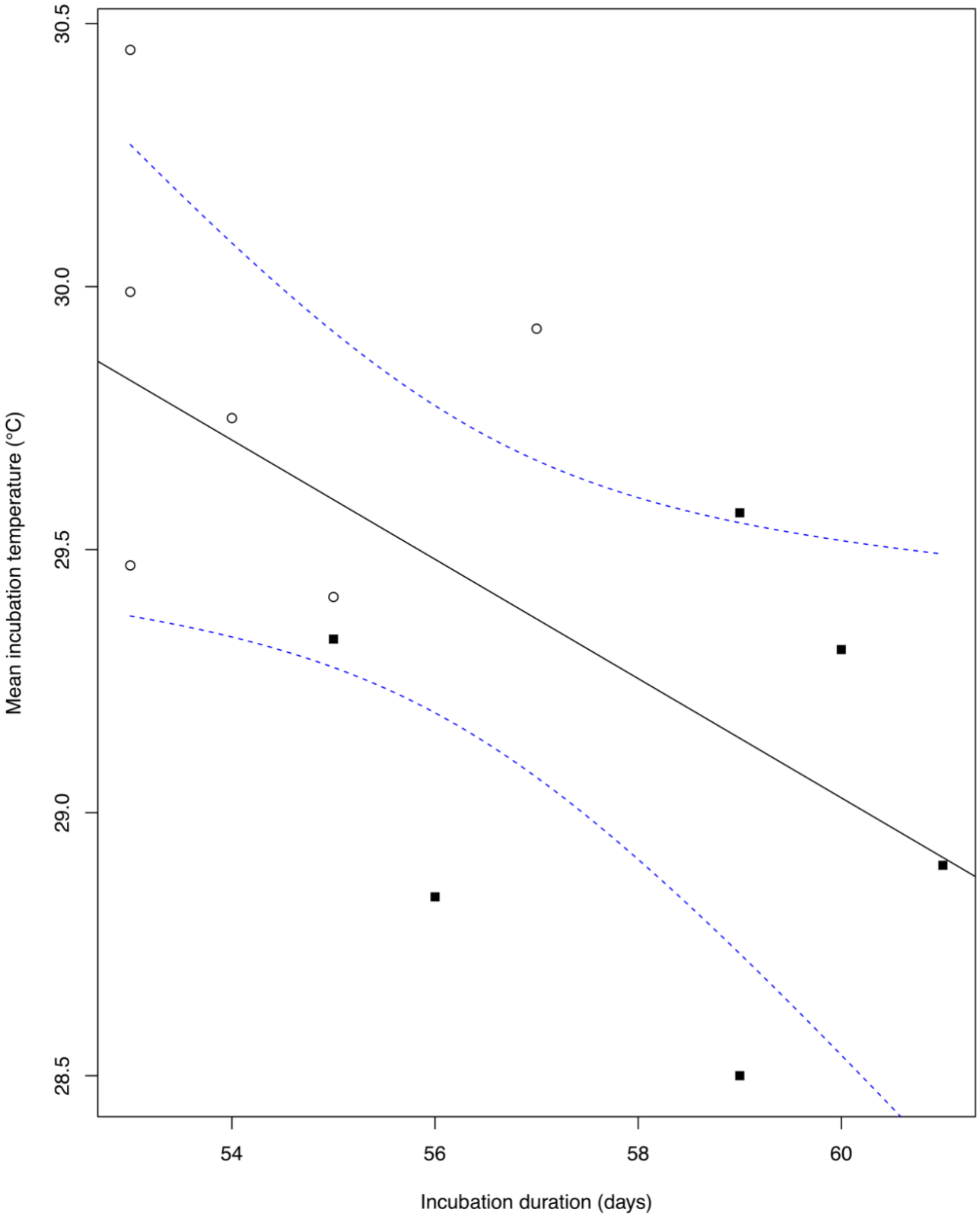

Figure 4.2: The relationship between incubation duration and incubation temperature for nests in this study. The dashed blue lines represent 95% confidence intervals, the filled squares represent wet nests and the unfilled circles represent dry nests. The equation for the relationship is mean incubation temperature (°C) = 35.8 – 0.11 × d, where d = incubation duration (days)

duration decreased from 80 days at 26°C to approximately 50 days at 32°C, with temperature explaining 95.7% of the variation in incubation duration (Matsuzawa *et al.*, 2002). Hatchlings from nests that had shorter incubation durations and were more likely to be from warm nests, had significantly higher CT_{max} than hatchlings from nests with longer incubation durations that were more likely to be cool nests. However, it remains unclear whether this effect is short-term or whether hatchlings incubated in warm nests retain a higher CT_{max} long-term. Additionally, without incubation temperature data, we cannot determine whether hatchling thermal tolerance is the result of acclimation to temperatures at the end of incubation or whether thermal tolerance is the result of developmental changes that occur throughout the entirety of incubation. Studies on adult organisms among taxa (Klok & Chown, 2003; Yang *et al.*, 2008; Zhang & Kieffer, 2014; Llewelyn *et al.*, 2017) have shown that CT_{max} is generally determined by the recent thermal conditions experienced by individuals. Further studies have shown that incubation temperatures did not have a significant effect on the thermal tolerance of adult lizards raised at a single temperature (Llewelyn *et al.*, 2018; Gunderson *et al.*, 2020) and studies that observed negative relationships between thermal tolerance and incubation temperatures tended to acclimate individuals before testing (Dayananda *et al.*, 2017; Llewelyn *et al.*, 2017). Therefore, it is likely that hatchlings in this study acclimated to nest temperatures during incubation and that an increased period of acclimation to cooler or warmer temperatures post-emergence would override the effects of incubation temperature (Yang *et al.*, 2008; Abayarathna *et al.*, 2019). We also considered the possibility that thermally tolerant hatchlings survive incubation, while less tolerant hatchlings do not. This would result in warm conditions producing fewer hatchlings that are more thermally tolerant and cool conditions producing more hatchlings, but the additional hatchlings from the cool nests would be less resilient to extreme temperatures. However, we did not observe a relationship between hatching success and thermal tolerance, suggesting that incubation temperatures do not select for thermally tolerant hatchlings. Expression of heat shock proteins (detailed below) increase embryonic thermal tolerance but decrease hatchling thermal tolerance, suggesting that thermally tolerant embryos may have reduced survival post-emergence rather than higher survival (Gao *et al.*, 2014).

The role of acclimation may also explain the differences in CT_{max} between our study and that of Drake and Spotila (2002), who measured the critical thermal maximum of green sea turtle hatchlings from Playa Grande, Costa Rica. In our study, hatchling CT_{max} was 40.19°C

compared to 41.3°C for hatchlings from Costa Rica. Costa Rican hatchlings had a mean initial body temperature of 29.7°C compared to 29.04°C (dry hatchlings) and 28.73°C (wet hatchlings) in our study. Malaysian hatchlings in our study are likely to have been acclimated to lower temperatures, as shown by the differences in initial body temperature and because our hatchery was shaded. This potentially explains the reduced ability of hatchlings from our study to tolerate extreme temperatures as shown by their lower CT_{max}. Beach characteristics are vitally important, with the differences in nest temperature among studies possibly resulting from differences in nest depth, sand type and colour, shading, nest location and differences in climate between the two nesting beaches (Kaska *et al.*, 1998; Hays *et al.*, 2001; Hill *et al.*, 2015). Alternatively, the fact that one study tested CT_{max} in air and the other in water may have also led to differences in thermal tolerance, since hatchlings may be more tolerant of elevated temperatures in water than in air. Lastly, the observed variation in thermal tolerance between this study and Drake and Spotila (2002) may reflect genetic differences between these two geographically separate populations. Costa Rican nesting beaches may be hotter than Malaysian beaches leading to Costa Rican green sea turtle hatchlings naturally exhibiting greater thermal tolerance.

Current research attributes differences in thermal tolerance to varying expression of heat shock proteins, both within and among species (Gehring & Wehner, 1995; Moseley, 1997; Basu *et al.*, 2002; Carmel *et al.*, 2011). Higher temperatures and longer exposures to these temperatures result in increased expression of heat shock protein genes (Tedeschi *et al.*, 2015), with species from warmer regions producing more heat shock proteins at any given temperature than species from cooler regions (Ulmasov *et al.*, 1992). Heat shock protein levels can remain elevated for days after heat shock (Lund *et al.*, 2003), potentially in preparation for further heat stress events. While moderate heat shock protein production leads to increased thermal tolerance, excessive production can reduce tolerance (Krebs & Feder, 1998) potentially by interfering with cell function (Feder & Hofmann, 1999). Overexpression of heat shock protein genes during embryonic development can lead to increased embryonic thermal tolerance but also to decreased hatchling thermal tolerance post-emergence (Gao *et al.*, 2014). The warmer incubation temperatures of dry nests in our study may have led to hatchlings from those nests experiencing increased heat shock protein production. Considering that the relationship between total protein and CT_{max} was weak, our findings suggest that hatchling hydration has a limited role in determining thermal tolerance, while heat shock protein production or efficacy may be limited in individuals with higher total

protein concentrations (Dill *et al.*, 2011). However, previous studies have found that extreme levels of dehydration can alter thermal tolerance in reptiles (Plummer *et al.*, 2003). Thus, hydration may influence sea turtle hatchling thermal tolerance, although not at the levels we measured.

Maternal effects can also have considerable influence on hatchling traits (Brooks *et al.*, 1991; Wallace *et al.*, 2006; Andrews, 2018). While the influence of maternal effects on hatchling morphology is well established in reptiles and birds (Finkler, 1999; Radder *et al.*, 2004; Wallace *et al.*, 2006), its role in determining other hatchling traits, such as sex, is less certain (Radder, 2007). Maternal identity may influence thermal tolerance genetically (Urban *et al.*, 2014) or by altering yolk quantity and quality (Warner & Lovern, 2014). In our study, nest ID explained considerable variation in thermal tolerance (21.7%), PCV (31.4%) and TP (25.2%) with moisture treatment. While this suggests that maternal identity is playing an important role in determining hatchling thermal tolerance, the mechanisms behind this effect require further investigation. In particular, future studies should investigate the potential effects of yolk and albumin composition (i.e. relative protein and lipid concentrations) and genetics on total protein, specifically heat shock proteins.

Currently, shading and watering nests are popular management techniques for decreasing nest temperatures and minimising embryonic mortality on nesting beaches (Hill *et al.*, 2015). While this may decrease nest temperatures and maximise hatching success, it could have negative repercussions for hatchlings during emergence and dispersal. The decreased nest temperatures caused by higher moisture levels or increased shade could lead to the production of hatchlings with lower thermal tolerance that may have to crawl across hot sand to reach the ocean. This could shift mortality events from inside the nest during development to the beach surface during emergence and dispersal, instead of increasing hatchling recruitment. However, the upper thermal limit of developing embryos (35°C) is considerably less than the CT_{max} of hatchlings (40.19°C in our study), suggesting that embryonic mortality is likely to become problematic before hatchling mortality. Additionally, hatchlings generally emerge during the night when sand temperatures are cooler, although some nests do emerge during the day or early evening when surface sand is still hot (Witherington *et al.*, 1990). Future management interventions involving watering or shading nests may therefore require reduced nest temperatures to maximise hatching success, yet may also result in increased mortality of hatchlings during dispersal, particularly in nests that emerge during the day. The

negative effects of this trade-off will be minimal in projects that guard hatchlings after they are released from hatcheries but may reduce hatchling survival when these management interventions are made on natural beaches and nests. Additionally, the negative effects of reduced thermal tolerance on hatchling survival will be minimal in nests that emerge at night or only disperse a short distance from nest to ocean.

Current projections suggest that embryonic mortality is the largest threat to sea turtle populations globally (Laloe & Hays, 2017; Montero *et al.*, 2018a; Montero *et al.*, 2018b; Monsinjon *et al.*, 2019). These projections do not generally consider hatchling mortality on the nesting beach and therefore, the number of hatchlings projected to survive incubation may be much higher than the number of hatchlings that actually make it the ocean. As sand temperatures continue to warm, the number of hatchlings surviving dispersal from the nest to the ocean may decrease. However, if hatchling thermal tolerance increases with warmer sand temperatures, the discrepancy between the number of hatchlings that successfully hatch and that enter the ocean may not increase as rapidly as previously thought. Although hatching success is a key indicator of population viability, the number of hatchlings that successfully hatch becomes irrelevant if few or none of those hatchlings are physiologically capable of surviving post-emergence. Future projections should consider not only embryonic thermal tolerance under future sand and nest temperatures but also hatchling thermal tolerance, in order to refine current estimates of hatchling recruitment and survival.

In conclusion, our study showed that moisture concentrations during incubation did not directly influence hatchling hydration or thermal tolerance. Rather, moisture levels altered nest temperatures and it was nest temperature that determined hatchling thermal tolerance. Hatchlings acclimated to nest temperatures, with warmer nests producing hatchlings with higher CT_{max}. Hatchling hydration and body size also influenced thermal tolerance, although both relationships were weak and require further investigation. Future studies will need to consider how a wider range of temperatures influence thermal tolerance, particularly at temperatures near the 35°C upper thermal limit for embryos. Furthermore, future studies should investigate at what stage during incubation temperature influences thermal tolerance, and whether temperature effects can be overridden by acclimating hatchlings post-emergence or acclimating embryos during the final days of incubation.

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Chapter 5. Ontogeny and ecological significance of metabolic rates in sea turtle hatchlings

Preparing a turtle for processing while on a field trip during my time in Hawaii collecting additional metabolic rate data from T Todd Jones.

Photo taken by Cam Allen.

Submitted to *Functional Ecology*

5.1 ABSTRACT

Sea turtle hatchlings must avoid numerous predators as they disperse from their nesting beaches to foraging grounds. In order to minimise the time spent in predator-dense neritic waters, hatchlings experience the 'frenzy period', characterised by almost continuous swimming for approximately the first 24 hours post-emergence. Post-frenzy, hatchling activity gradually declines as they swim towards foraging grounds in less predator-dense pelagic waters. Despite this decrease in predator density, hatchlings still face threats including starvation if they cannot reach foraging grounds before depleting yolk reserves. Of particular importance during the frenzy and post-frenzy periods are metabolic rates that determine the ability of hatchlings to fuel dispersal activities and behaviour. It has been well-documented that during the frenzy, hatchlings exhibit elevated metabolic rates to power their almost continuous swimming and hyperactivity, but studies on the post-frenzy metabolic rates of hatchlings and the differences among species are sparse. Thus, we measured the frenzy and post-frenzy oxygen consumption of five species of sea turtle hatchlings at different activity levels and ages in order to compare the ontogeny of sea turtle hatchling metabolic rates. Metabolic rates at different activity levels and behavioural stages varied significantly, but maximal metabolic rates were always higher than resting metabolic rates. Interestingly, metabolic rates during routine swimming were often similar to resting metabolic rates. Crawling metabolic rates did not differ among species, potentially indicating the use of anaerobic energy pathways by hatchlings during the crawl to the water. Green sea turtle hatchlings had the highest oxygen consumption during routine and maximal swimming during frenzy and post-frenzy periods. In comparisons, leatherback hatchlings exhibited elevated resting metabolic rates and lower metabolic rates during routine swimming than the cheloniids. The differences in metabolic rate reflect the varying dispersal stratagems of each species. Variation in metabolic rates has important implications for hatchling dispersal ability, hatchling growth, yolk consumption and therefore, hatchling survival and population dynamics.

5.2 INTRODUCTION

The majority of oviparous reptilian species provide minimal parental care to their offspring (Somma, 1990). Thus, offspring must emerge from the nest and disperse on their own. Consequently, smaller and slower offspring may be at greater risk of predation than offspring that are larger and faster (Janzen *et al.*, 2000; Pilcher *et al.*, 2000; Cavallo *et al.*, 2015). Sea

turtle hatchlings have high mortality rates compared to other reptilian species because they experience high rates of predation during their prolonged dispersal. In particular, predation rates are highest when the hatchlings crawl from the nest to the ocean and as the hatchlings swim in near-shore waters (Gyuris, 1994). Hatchlings that crawl or swim more slowly spend more time on the beach and in neritic waters and are thus more susceptible to predation than hatchlings that are faster crawlers or swimmers (Whelan & Wyneken, 2007). To minimise the time spent in predator-dense zones, hatchlings undergo a period of hyperactivity for approximately the first 24 h post-emergence. During this period of hyperactivity termed the 'frenzy' (Carr, 1962), hatchlings swim almost continuously and exhibit high thrust production (Wyneken & Salmon, 1992; Booth, 2009) as they quickly disperse from the natal beach and surrounding waters.

While an effective strategy for predator evasion, the continuous swimming and high thrust production of the 'frenzy' is energetically demanding (Wyneken, 1997; Jones *et al.*, 2007; Booth, 2009). During the first 24 h of the frenzy, hatchling swimming activity can be broken into three phases: the rapid fatigue phase when oxygen consumption is initially high and quickly declines, followed by the slow fatigue phase when oxygen consumption rates continue to drop, but at a slower rate, and lastly the sustained effort phase when oxygen consumption is relatively stable (Booth, 2009). As most hatchlings survive solely on residual yolk reserves during dispersal, maintaining high activity levels places hatchlings at greater risk of fatigue and resource depletion before reaching foraging grounds compared to hatchlings with lower energy demands (Kraemer & Bennett, 1981; Jones *et al.*, 2007). Thus, hatchling activity levels are highest during the initial dispersal across the beach and through neritic waters where predator-densities are highest (Salmon & Wyneken, 1987; Wyneken & Salmon, 1992). Once hatchlings enter deeper, pelagic waters, the total time that they spend swimming per day gradually decreases (Salmon & Wyneken, 1987; Wyneken & Salmon, 1992), although sea turtle species differ in the rate at which they shift their swimming activity and behaviour (Wyneken & Salmon, 1992). These differences are often attributed to variation in life history among species. For example, flatback hatchlings remain completely within neritic waters during dispersal and they exhibit smaller reductions in swimming activity levels compared to other species, potentially in order to avoid predators in these predator-dense waters (Salmon *et al.*, 2009). Differences in swimming activity have also been observed among populations, providing further support that divergence in life history and selective pressures drive variation in swimming activity (Wyneken *et al.*, 2008).

While the ontogeny of swimming activity (i.e., the change in swimming behaviour as hatchlings age) between frenzy and post-frenzy swimming has been studied previously (Salmon & Wyneken, 1987; Wyneken & Salmon, 1992; Burgess *et al.*, 2006; Booth, 2009; Salmon *et al.*, 2009; Sim *et al.*, 2015), the ontogeny of metabolic rates remain relatively unstudied (Wyneken, 1997; Jones *et al.*, 2007). This difference is likely because hatchling metabolic rates (MRs) are typically measured by estimating oxygen consumption, which requires specialised equipment. More common are proxies of metabolic rate that include direct measures of swimming behaviour, flipper stroke rates, and swimming bout durations. However, metabolic rates are key measures of the energetic capacity of hatchlings to disperse, determining how long they can remain active. Hatchlings that have higher metabolic rates may have a greater ability to swim quickly, but also may consume their yolk reserves more quickly than hatchlings with lower metabolic rates. Determining how sea turtle hatchlings utilise energy is critical for understanding limits of hatchling dispersal, foraging and growth, which has important implications for population dynamics and ecology. Previous studies showed that the ontogeny of hatchling oxygen consumptions varies among species (Wyneken, 1997; Jones *et al.*, 2007), but studies comparing the ontogeny of metabolic rates in sea turtle hatchlings are few. Here, we measured and compared the metabolic rates of five sea turtle species during the frenzy and post-frenzy. We measured oxygen consumption during rest (resting metabolic rate, RMR) when hatchlings were quiescent, crawling metabolic rate (CMR) when hatchlings were actively and continuously crawling on sand, routine swimming (active metabolic rate, AMR) when hatchlings were actively and continuously swimming of their own volition, and maximal metabolic rate (MMR) when hatchlings were being stimulated to swim with maximum effort. Each measure reflects specific energy requirements to support the various ecological demands during the frenzy and post-frenzy phases: RMR reflects the energy requirements to support breathing and other basic physiological functions such circulating blood (Willmer *et al.*, 2009); CMR represents the energy requirements to fuel hatchling dispersal from the nest to the ocean; AMR represents normal activity associated with foraging and general locomotion (Wallace & Jones, 2008); and MMR represents the maximum energy production capable by an individual turtle, such as when threatened by a perceived predator (Jones *et al.*, 2007; Wallace & Jones, 2008). We measured oxygen consumption to compare differences in metabolic rates among behavioural stages, activity levels and species. Additionally, we compared each species' aerobic scopes. We hypothesised that metabolic rates and aerobic scopes vary among activity

levels, behavioural stages and species in a manner that matches the species' and population's early life history stratagems. Specifically, we hypothesised that species with greater predation pressure during the frenzy would exhibit higher metabolic rates during the frenzy than species with lower predation pressures. We also hypothesised that post-frenzy, species with extended dispersal migrations would exhibit higher metabolic rates than species, such as flatbacks, that undertake shorter dispersal migrations. We aimed to then evaluate any differences in the context of the life history patterns and ecology.

5.3 METHODS

In this study we used two methods for measuring turtle oxygen consumption: closed and open flow respirometry (Table 5.1). Closed respirometry requires creating a chamber with a constant volume and circulating air from the chamber containing the animal through the oxygen analyser and back into the chamber. As oxygen cannot enter this closed system, it is possible to record the drop-in oxygen within the chamber as the animal consumes the available oxygen. Open flow respirometry draws air continuously from an external source, generally the atmosphere or from a tank, through the chamber containing the animal, then through the oxygen analyser before expelling the air back into the atmosphere. By comparing the concentration of oxygen in the air entering and exiting the chamber, it is possible to calculate the oxygen consumption of that animal. Open flow systems allow for measuring metabolic rates over longer time periods because there is a continual flow of oxygen into the chamber throughout testing. We measured metabolic rate in turtles that were resting (RMR), crawling (CMR) and swimming, both routinely (AMR) and maximally (MMR). Turtles were defined as resting when stationary (only breathing) within the respirometry chamber. Turtles were defined as crawling when actively moving around an empty, dry respirometry chamber . Swimming turtles were considered to be swimming either routinely or maximally: routine swimming (AMR) was assigned when turtles swam without encouragement or prodding, and maximal swimming (MMR) was assigned when turtles were tapped on the carapace with a piece of wire to mimic a predation event under natural conditions (Jones et al., 2007).

Table 5.1: Summary of the methodology used to test each species' oxygen consumption and the behavioural stage at which each species was tested. We list the activity level that was measured for each species, behavioural stage and technique

	Closed respirometry (2017/18)		**Closed respirometry (2010)**		**Open flow respirometry (1996 & 2000)**	
	Frenzy	**Post-frenzy**	**Frenzy**	**Post-frenzy**	**Frenzy**	**Post-frenzy**
Flatback	RMR & MMR	RMR & MMR				
Green	RMR & MMR		AMR		RMR, CMR & AMR	RMR & AMR
Olive Ridley	RMR & MMR	RMR & MMR				
Leatherback				AMR	RMR, CMR & AMR	RMR & AMR
Loggerhead				AMR	RMR, CMR & AMR	RMR & AMR

5.3.1 Closed respirometry: flatback, green and olive ridley sea turtle hatchlings

We collected olive ridley (*Lepidochelys olivacea*) and flatback sea turtle (*Natator depressus)* eggs in Australia from the Tiwi Islands, NT and Curtis Island, QLD in 2017 and 2018, respectively. We then transported the eggs to Monash University, Melbourne, VIC where they were placed into incubators (1602-N Hovabator).

Green sea turtle eggs *(Chelonia mydas)* were collected from Kijal beach, Malaysia, 42km from the Lang Tengah Turtle Watch hatchery in 2018. The eggs were transported to the shaded hatchery in buckets lined with sand and buried in the centre of a $1m_2$ plot with the bottom of the nest at a depth of 70cm.

After emerging from the eggs, olive ridley and flatback hatchlings were given 48 hours to internalise their yolk sac. We then marked hatchlings on the carapace with unique patterns using non-toxic nail polish and measured hatchling mass using electronic scales (±0.001g).

We measured both resting (RMR) and maximal metabolic rate (MMR) of hatchlings. First, we tested RMR by placing hatchlings in a small closed chamber (~375mL) with an O_2 probe (PASCO PS-6524) recording the change in O_2 concentration. We used soda lime (Scharlau, Australia) and Drierite™ (Hach, Australia) to remove CO_2 and H_2O from the air, respectively. We calibrated the O_2 probe to the ambient O_2 concentration (20.9%) before each trial began, and checked the system for leaks using N_2 gas. We began RMR trials once the hatchling became still (generally within 5 min) and restarted trials if the hatchling became active or agitated. Hatchlings remained in the respirometry chamber for 20 min. Olive ridley and flatback hatchlings were tested in a controlled temperature room at 25°C, while green hatchling testing occurred in the Lang Tengah Turtle Watch headquarters at ambient temperature (27.5 ± 1.2°C). Oxygen consumption was calculated by subtracting the O_2 concentration at the end of each trial from the concentration at the start of each trial.
Next, we tested hatchling MMR when hatchlings swam maximally. We placed a glass chamber upside-down in seawater, creating a pocket of air between the water and the chamber (~1000mL). We pumped air from the chamber at ~200 ml min^{-1} over an O_2 probe (PASCO PS-2126A) sampling at 2Hz before returning the air to the chamber. The air was scrubbed using soda lime to remove CO_2 and drierite to remove H_2O before passing over the O_2 probe. Hatchlings were placed in elasticised harnesses and tethered to the top of the chamber with fishing line so they could swim but not touch the sides of the chamber. Trials lasted 15 min and to ensure the hatchlings swam maximally, we tapped them on the back of the carapace using a bent piece of wire passed underneath the chamber, encouraging a flight response (Jones *et al.*, 2007). Water temperatures for maximal metabolic rates were 26.3 ± 0.4°C for flatback and olive ridley hatchlings, and 26.6 ± 1°C for green hatchlings.

Olive ridley hatchlings were tested during the frenzy (0 weeks of age, sample size (N)=74, mass ± se 16.46 ± 0.21g) and post-frenzy (4 weeks of age, N=70, 19.39 ± 0.28g), green hatchlings were tested during the frenzy only (N=95, 21.37 ± 0.21g) and flatback hatchlings were tested during the frenzy (N=80, 40.39 ± 0.31g) and post-frenzy (N=79, 63.32 ± 0.52g).

Olive ridley and flatback hatchlings were housed under a day/night cycle of 12 hours and, maintained between 26 and 27°C.

After testing was completed, 4-week-old olive ridley and flatback hatchlings were transported back to the site of collection and released. Green hatchlings were released on the beach adjacent to the Lang Tengah Turtle Watch hatchery within 24 hours of emerging. Eggs were collected under Queensland scientific purposes permit WITK18685417 (flatbacks), Northern Territory permit to take wildlife 62703 (olive ridleys) and Terengganu State Fisheries Office approval to carry out research work SEATRU/RES/17/01 (greens). Experimental procedures were conducted under approval SEATRU/RES/17/01 for green sea turtles and under Victorian research permit 10008208 for flatback and olive ridley hatchlings. All procedures were approved by the Monash University School of Biological Sciences Animal Ethics Committee (approval BSCI/2018/08 for green sea turtles and BSCI/2016/23 for olive ridley and flatback sea turtles). Egg collection and hatchling release of olive ridley hatchlings was conducted with the permission and assistance of the Tiwi Land Council and the Science Reference Council.

5.3.2 Closed respirometry- leatherback, loggerhead and green sea turtle hatchlings

Hatchlings were collected from natural nests laid in Boca Raton, Florida, USA throughout June, July and August of 2010. Hatchlings were housed at Florida Atlantic University in clutch-specific tanks with separate water and filter systems for each clutch. Tank water was approximately the same temperature as ocean water and all tests were conducted at 24°C-28°C. Hatchlings were released offshore following testing.

Testing occurred in a 35cm × 35cm Plexiglass™ respirometry chamber or a glass and acrylic chamber (loggerheads and leatherbacks) that was 50.8cm × 25.4cm. Chambers were filled with seawater so that an air space of 1-2cm in height was left between the chamber lid and the water. Thus, the air volume during testing could be calculated from the chamber cross-sectional area and the height of the air space. Air from inside the chamber was pumped through an Applied Electrochemistry O_2 Analyser S-3A (AEI Technologies, Pittsburgh, PN, USA) and recirculated back into the chamber. We replaced the seawater with fresh, autoclaved seawater allowed to come to room temperature between clutches

Hatchlings were randomly selected from each clutch for testing. Leatherback hatchlings were tested at 20 days (sample size (N)=4, mass ± se 68.02 ± 5.47g), 23 days (N=6, 61.56 ± 3.32g) and 44 days (N=1, 99.21g). Loggerhead hatchlings were tested at 6 days (N=5, 16.81 ± 0.23g), 43 days (N=2, 60.68 ± 7.95g), 51 days (N=2, both 89.87) and 52 days (N=1, 53.65g). Green turtle hatchlings were all tested on the day of emergence (N=6, 24.6 ± 0.18g). Tank temperature was recorded before each trial (range: 24-30°C). Leatherback hatchling testing lasted for an average of 55 min, green hatchlings for 20 min and loggerheads for an average of 27 min.

Hatchling collection, testing and housing were conducted under FAU IACUC protocol A10-18 and Florida Sea Turtle Permit #073.

5.3.3 Open flow respirometry- leatherback, loggerhead and green sea turtle hatchlings

Green, loggerhead, and leatherback turtle hatchlings were collected from natural nests laid in Boca Raton, Florida USA throughout June, July and August of 1996 and 2000. Additional leatherback turtle hatchlings were collected from natural nests laid in Hillsboro Beach, Juno Beach, and Jupiter Beach, Florida USA during the same time periods. Hatchlings were housed at Florida Atlantic University in clutch-specific tanks with separate water and filter systems for each clutch. Tank water was approximately the same temperature as the ocean water and all tests were conducted at 24°C-28°C. Hatchlings were released offshore following testing.

When measuring resting metabolic rates, hatchlings were placed in a black container (10 cm × 7.5 cm, approximately 470mL) closed with a large rubber stopper fitted with air intake and outflow. Each turtle was allowed to acclimate for 30 min, and hatchling movement was minimised in the small container. Once hatchlings were inactive (based on no audible sound from the claws or flippers on the glass), we closed the container, began measuring the O_2 consumption and measured for 90 min. If hatchlings became active, we restarted metabolic measurements.

For measurements of metabolic rates during crawling (CMR) and routine swimming metabolic rate (AMR), testing occurred in a 26 L tank fitted with an acrylic respirometry

chamber and sealed with petroleum jelly. During CMR testing, hatchlings were allowed to crawl on a textured glass floor. During testing of routine swimming metabolic rate, hatchlings were allowed to swim of their own volition, without encouragement. The chamber was filled with seawater so that an air pocket of 2cm in height × 25 cm × 20 cm was left between the chamber lid and the water. Thus, the air volume during testing could be calculated following Withers (1977). Air was drawn from the chamber and passed through an Applied Electrochemistry O_2 Analyser S-3A (AEI Technologies, Pittsburgh, Pennsylvania USA) before being pumped into the atmosphere. Between turtles, we sanitised the tank and replaced the seawater with fresh, autoclaved seawater at room temperature.

Hatchlings were randomly selected from each clutch for testing. All were weighed using an electronic balance or a Pesola™ scale. Leatherback, loggerhead and green hatchlings were tested during the frenzy (sample size ($N_{loggerheads}$)=21, mass ± se 18.39 ± 0.37g; N_{greens}=24, 24.72 ± 0.36g; $N_{leatherbacks}$=25, 44.89 ± 0.72g) and post-frenzy ($N_{loggerheads}$=28, 22.14 ± 1.06g; N_{greens}=33, 35.6 ± 1.48g; $N_{leatherbacks}$=25, 59.03 ± 2.58g). Hatchlings were allowed to acclimate for 30 min. Room temperature was recorded before each trial (23.61 ± 1.5°C). For resting and active metabolic rate, hatchlings were tested for 90 min, while for crawling metabolic rate hatchlings were tested for 40 min.

Hatchling collection, testing and housing were conducted under Florida Sea Turtle Permit 073.

Detailed descriptions of egg collection, transport, incubation, hatchling housing and respirometry techniques can be found in appendix II (p256).

5.3.4 Data analysis

For closed system respirometry, we calculated oxygen consumption (VO_2) (μL min^{-1}) using the formula:

(1)

where $\%O_{2I}$ is the initial percentage of oxygen in the respirometer at the start of the trial, $\%O_{2F}$ is the final percentage of oxygen in the respirometer at the end of the trial, V is the volume of air contained by the respirometer (μL), t_I is the time at the start of the trial (min)

and t_F is the time at the end of the trial (min). When calculating the mass-specific metabolic rates of hatchlings, we used a mass exponent of 0.67 (Ultsch, 2013) to correct for allometric relationships between metabolic rate and hatchling mass.

For open flow respirometry, we calculated oxygen consumption (µL min^{-1}) using the formula:

(2) where FR is the flow rate (µl/min) of air through the chamber, $\%O_{2I}$ is the incoming fraction of oxygen in the air entering the chamber and $\%O_{2E}$ is the fraction of oxygen in the air exiting the chamber. Oxygen consumption was calculated every 5 min and then averaged to calculate the mean oxygen consumption over the entire trial.

To determine the overall differences in metabolic rate at all activity levels, behavioural stages and species, we used a linear mixed effects model of mass-specific metabolic rate using in the lme4 package in R (Bates *et al.*, 2014; R Core Team, 2014). We chose mixed effects models to account for our repeated measures of individual hatchlings and for our unbalanced experimental design. Activity (resting, crawling, routine and maximal swimming), behavioural stage (frenzy and post-frenzy) and species (green, leatherback, loggerhead, olive ridley and flatback) were the fixed effects, while hatchling ID nested within species was the random effect. We included interaction terms for all fixed effects to account for changes in metabolic rate that were dependent on two or more variables (i.e. the change in metabolic rate from frenzy to post-frenzy by species or by activity level).

Our data were not normally distributed, so we ran our linear mixed effects model with a log link function to meet the assumption of normality. All of our fixed effects and interactions were significant, so we explored each fixed effect separately to identify differences between each level of that effect. We constructed pairwise comparisons using Tukey tests in the package ‘emmeans’ to explore each fixed effect separately.

Aerobic scopes represent the ability of an organism to increase its metabolic rate above resting metabolic rate (Jackson & Prange, 1979; Jones *et al.*, 2007). True aerobic scopes are determined from maximal and standard metabolic rates (SMR) in ectotherms (basal for endotherms). SMR is defined as the metabolic rate of an ectotherm with no muscular activity

and is not actively digesting food, at a specified temperature (Nagy, 2000). However, sea turtle hatchlings utilise yolk reserves for up approximately a week post-hatching. Thus, it is not possible to measure SMR in hatchlings with yolk reserves e.g. sea turtles. Therefore, we calculated factorial aerobic scopes by dividing MMR by RMR to show ontogenetic differences among species in their ability to increase their metabolic rate above resting levels for dispersal, escaping predation and for chasing prey. Measurements of RMR include the costs of maintenance i.e. SMR, the costs of digestion and the costs of somatic growth.

We examined aerobic scope between behavioural stages using linear mixed effects models to identify differences among species. Behavioural stage and species were the fixed effects and hatchling ID nested within species was the random effect. We constructed pairwise comparisons using Tukey tests in the package 'emmeans' to identify how fixed effects differed. Our level of significance was 0.05.

5.4 RESULTS

5.4.1 Overall variation in metabolic rates with activity level, behavioural stage and species

The metabolic rates we measured using different respirometry techniques were consistent for animals at the same activity levels, species and behavioural stage. Thus, respirometry technique was not a confounding factor, allowing us to pool the results from each technique into the single dataset used here (Table 5.2).

Hatchling metabolic rates varied significantly with behavioural stage, activity and species (Table 5.3). Hatchling ID nested within species explained 99.9% of the variance in metabolic rate. The interactions between activity and species, activity and behavioural stage, species and behavioural stage and among all three fixed effects were significant. Thus, we also evaluated differences among and within species, activity and behavioural stage separately. We report the results of mass-specific metabolic rate comparisons below.

5.4.2 Change in oxygen consumption between behavioural stages

Within the activity analyses, RMR (i.e. when hatchlings were quiescent), did not differ between the frenzy and post-frenzy in loggerhead ($z=-0.863$, $p=0.388$), olive ridley ($z=0.689$, $p=0.491$) and green hatchlings ($z=-1.832$, $p=0.067$). However, flatback ($z=4.765$, $p<0.0001$)

Table 5.2: Olive ridley, flatback, leatherback, loggerhead and green sea turtle hatchlings resting metabolic rate (RMR), crawling metabolic rate (CMR), metabolic rate during routine swimming (AMR) and maximal metabolic rate (MMR) during the frenzy and post-frenzy. Values are given as μL O_2 min^{-1} (whole animal) and μL O_2 $g^{-0.67}$ min^{-1} (mass-specific) ± standard error.

		Whole animal					**Mass-specific**				
		Olive ridley (μL O_2 min^{-1})	**Flatback (μL O_2 min^{-1})**	**Green (μL O_2 min^{-1})**	**Leatherback (μL O_2 min^{-1})**	**Loggerhead (μL O_2 min^{-1})**	**Olive ridley (μL O_2 $g^{-0.67}$ min^{-1})**	**Flatback (μL O_2 $g^{-0.67}$ min^{-1})**	**Green (μL O_2 $g^{-0.67}$ min^{-1})**	**Leatherback (μL O_2 $g^{-0.67}$ min^{-1})**	**Loggerhead (μL O_2 $g^{-0.67}$ min^{-1})**
Frenzy	RMR	30 ± 2.06	122.54 ± 4.5	79.2 ± 3.4	313.55 ± 35.93	63.73 ± 6.45	4.59 ± 0.31	10.3 ± 0.38	10.17 ± 0.45	23.76 ± 2.46	9.45 ± 1.06
	CMR			228.1 ± 95.85	377.09 ± 47.14	201.47 ± 32.28			26.62 ± 11.09	28.82 ± 3.33	28.19 ± 4.6
	AMR			445.17 ± 26.43	385.92 ± 36.87	253.19 ± 15.2			52.57 ± 3.34	30.84 ± 2.86	36.33 ± 2.24
	MMR	121.3 ± 6.88	280.93 ± 18.83	518.44 ± 14.46			18.42 ± 0.99	23.6 ± 1.56	66.68 ± 1.9		
	Mass (g)	16.46 ± 0.44	40.39 ± 0.43	22.17 ± 0.52	44.91 ± 0.52	18.39 ± 0.4					
Post-frenzy	RMR	26.89 ± 1.55	75.01 ± 1.82	156.09 ± 23.06	238.3 ± 28.95	131.32 ± 53.94	3.7 ± 0.22	4.67 ± 0.12	13.09 ± 1.94	13.94 ± 1.42	18.91 ± 8.61
	AMR			392.89 ± 68.98	235.21 ± 12.84	197.79 ± 29.82			37.24 ± 6.69	15.18 ± 0.97	19.65 ± 2.26
	MMR	78.98 ± 4.53	373.35 ± 18.52				10.83 ± 0.62	23.02 ± 1.08			
	Mass (g)	19.39 ± 0.53	63.32 ± 0.58	37.04 ± 1.95	63.45 ± 1.89	29.33 ± 3.81					

Table 5.3: Results from linear mixed effects model evaluating the effect of activity, behavioural stage, species and their interactions on oxygen consumption. Significant relationships are highlighted in bold.

	F-value	Df	p-value
Activity	**292.43**	**3**	**<0.001**
Behavioural Stage	**77.77**	**1**	**<0.001**
Species	**172.49**	**4**	**<0.001**
Activity: Behavioural Stage	**10.29**	**2**	**<0.001**
Activity: Species	**17.04**	**6**	**<0.001**
Behavioural Stage: Species	**6.14**	**6**	**<0.001**
Activity: Behavioural Stage: Species	**4.62**	**3**	**<0.001**

and leatherback hatchlings (z=2.121, p=0.034) all had higher RMR during the frenzy compared to post-frenzy (Figure 5.1). During routine swimming, when hatchlings were allowed to swim continuously of their own volition, all species had higher AMR during the frenzy compared to post-frenzy: loggerhead (z=3.827, p=0.0001), leatherback (z=4.303, p<0.0001) and green sea turtles (z=3.336, p=0.0008) (Figure 5.2). During maximal swimming, when hatchlings were encouraged to swim maximally, both olive ridley hatchlings (z=7.595, p<0.0001) and flatback hatchlings (z = 2.628, p=0.009) had higher MMR during the frenzy compared to post-frenzy (Figure 5.2), while the other species did not significantly differ from frenzy to post-frenzy.

5.4.3 The effect of activity level on oxygen consumption by species

During the frenzy, hatchling MMR was always higher than resting metabolic rate in green (Supplementary Table 5.1 (p264)), olive ridley (z=8.883, p<0.0001) and flatback sea turtle hatchlings (z=13.03, p<0.0001) (Figure 5.3). In post-frenzy olive ridley (z=5.28, p<0.0001) and flatback hatchlings (z=9.786, p<0.0001) MMR remained higher than RMR (Figure 5.4). During the frenzy, AMR was higher than RMR in loggerhead (z=-3.044, p=0.013) and green sea turtle hatchlings (Supplementary Table 5.1 (p264), Figure 5.3). Post-frenzy, the difference between AMR and RMR was maintained in green sea turtles (z=-4.409, p<0.0001), although not in loggerheads (z=-2.414, p=0.075) (Figure 5.4).

Figure 5.1: Resting mass-specific metabolic rate (μL O_2 min^{-1} $g^{-0.67}$) of sea turtle hatchlings during the frenzy and post-frenzy. Error bars represent standard error of the mean. Statistical differences between frenzy and post-frenzy resting metabolic rates within species are signified with *. Letters represent differences between species' resting metabolic rates during the frenzy and post-frenzy, respectively.

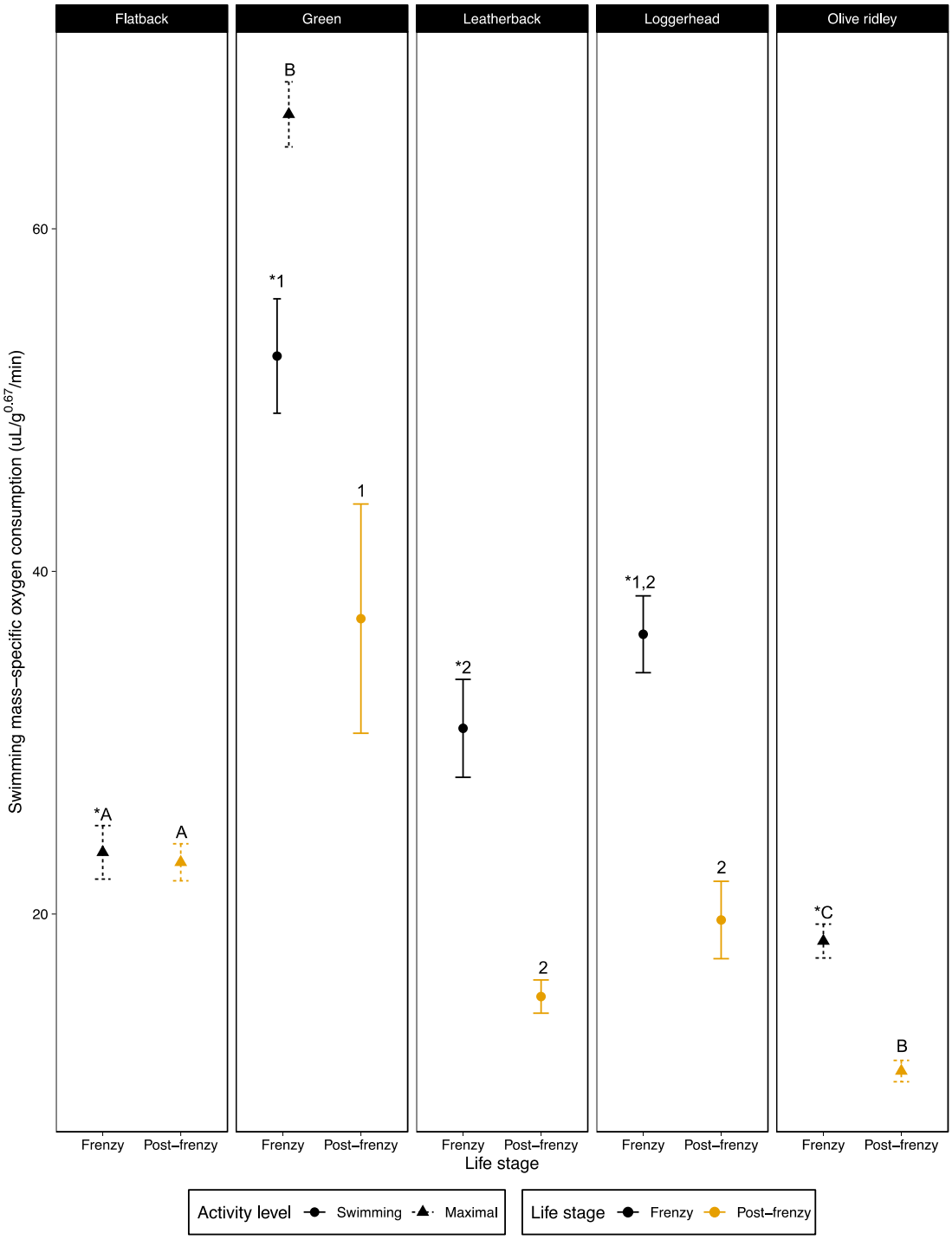

Figure 5.2: Mass-specific metabolic rate (μL O_2 min^{-1} $g^{-0.67}$) of swimming sea turtle hatchlings during the frenzy and post-frenzy. Error bars represent standard errors. We present measurements made during routine swimming (circles with solid error bars) and maximal swimming (triangles with dashed error bars). Statistical differences between frenzy and post-frenzy metabolic rates within species are signified with *. Numbers represent statistical similarities among species' routine swimming metabolic rates during the frenzy and post-frenzy, respectively. Letters represent statistical similarities among species' maximal metabolic rates during the frenzy and post-frenzy, respectively.

In leatherbacks, there was no difference between AMR and RMR during the frenzy (z=-0.947, p=0.78) or post-frenzy (z=-0.553, p=0.946). Additionally, neither in leatherbacks (z=1.252, p=0.594) nor loggerheads (z=2.226, p=0.116) did crawling metabolic rate (CMR) differ from RMR or from AMR (z=0.563, p=0.943; z=-1.868, p=0.242, respectively) during the frenzy (Figure 5.3).

CMR in green sea turtle hatchlings was higher than RMR, but lower than both MMR and AMR during the frenzy. However, green turtles did not differ between MMR and AMR during their frenzy (Figure 5.4, Supplementary Table 5.1 (p264)).

5.4.4 Inter-specific comparisons of metabolic rates

Species differed significantly in their metabolic rates during the frenzy. Leatherback hatchlings had the highest resting metabolic rate (RMR) and olive ridley hatchlings the lowest, while flatback and green hatchlings had intermediate RMR. Loggerhead hatchling RMR did not differ from any of the other species (Figure 5.1, Supplementary Table 5.2 (p265)).

Post-frenzy, green, leatherback and loggerhead hatchlings did not differ in their RMR, but all three species had significantly higher RMR than flatback and olive ridley hatchlings, that did not differ in their RMR (Figure 5.1, Supplementary Table 5.2 (p265)).

Green, leatherback and loggerhead sea turtle hatchling crawling metabolic rates (CMR) did not differ (Figure 5.5, Supplementary Table 5.3 (p266)).

While swimming routinely during the frenzy, the oxygen consumption of green hatchlings was higher than leatherback hatchlings, but loggerhead metabolic rates did not differ from the other species (Figure 5.2, Supplementary Table 5.4 (p266)). Post-frenzy, green hatchlings exhibited higher metabolic rates during routine swimming than loggerhead or leatherback hatchlings (Figure 5.2, Supplementary Table 5.4 (p266)).

When swimming maximally during the frenzy, green hatchlings had higher metabolic rates (MMR) than flatback hatchlings, and both that were higher than olive ridley hatchling metabolic rates (Figure 5.2, Supplementary Table 5.5 (p267)). During the post-frenzy swimming, flatback hatchlings had higher maximal metabolic rates (MMR) than olive ridley hatchlings (z=7.325, p<0.0001) (Figure 5.2).

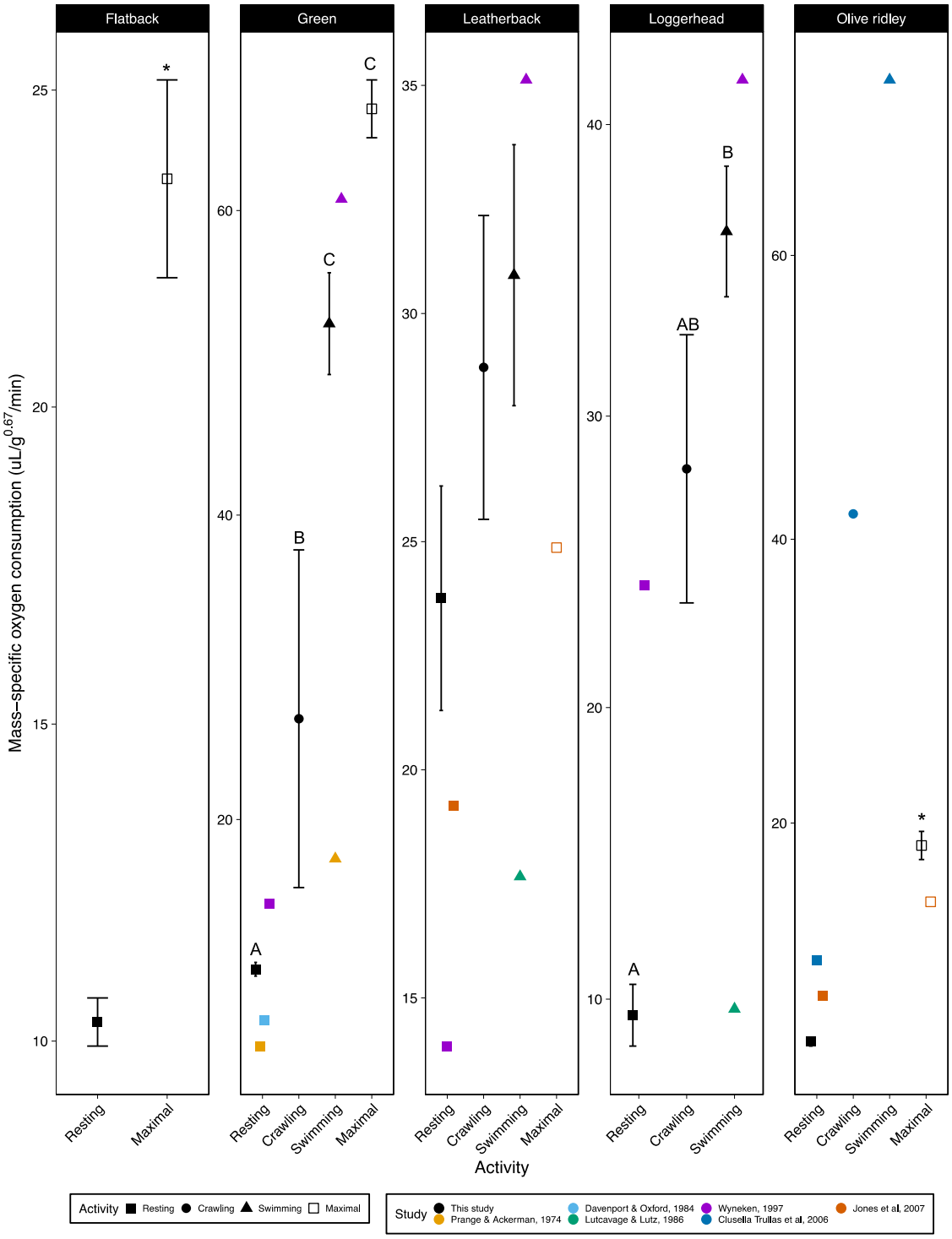

Figure 5.3: Comparison of sea turtle hatchling metabolic rates (μL O_2 min^{-1} $g^{-0.67}$) at different activity levels during the frenzy. Error bars represent standard errors and we also report data from earlier studies on hatchling metabolic rates. We denote statistical differences between two activity levels within species with * and between 3 or more activity levels with letters. We converted measurements in previous studies from a mass exponent of 1 to an exponent of 0.67 to correct for allometric relationships between metabolic rate and hatchling mass (Ultsch, 2013).

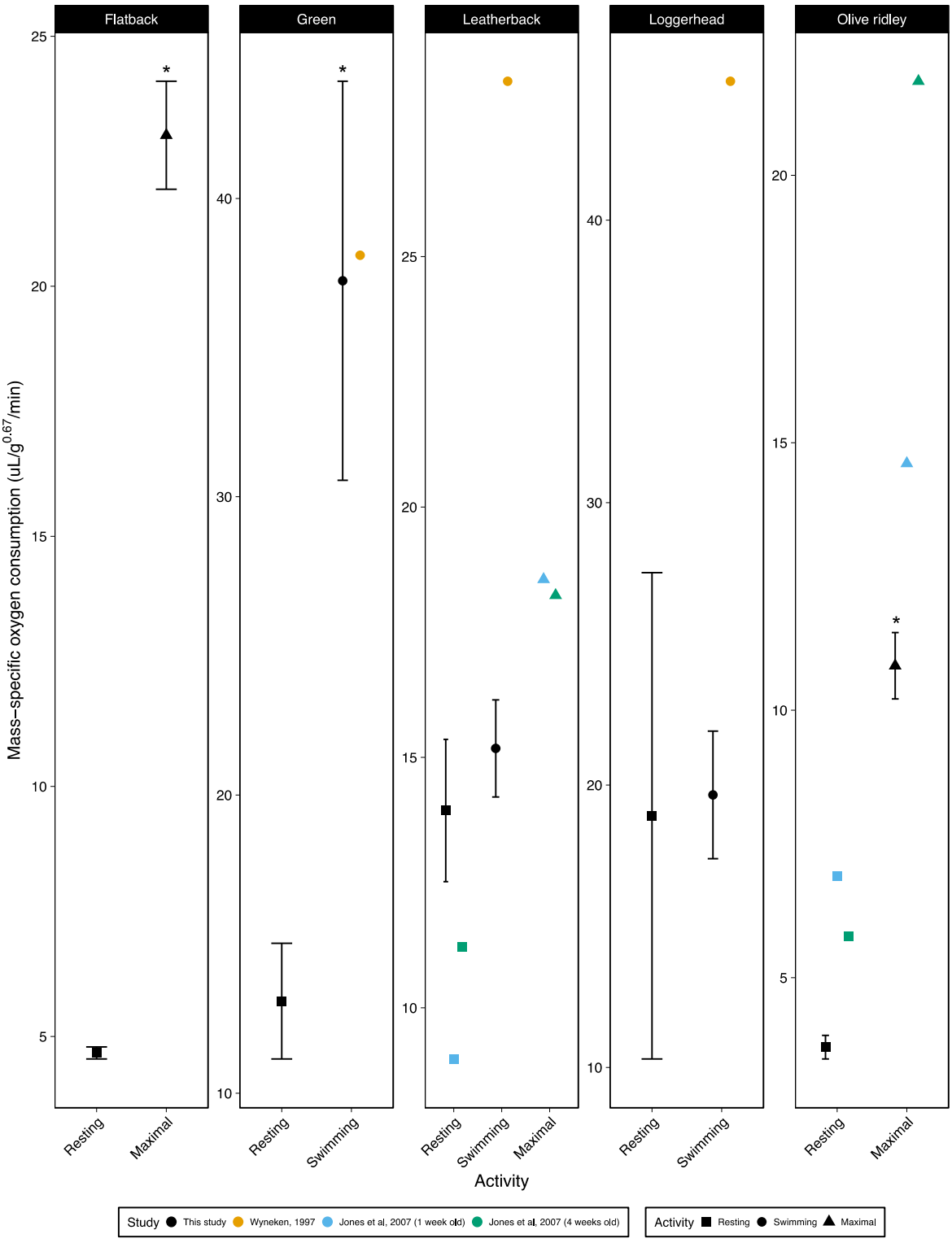

Figure 5.4: Comparison of sea turtle hatchling metabolic rates (μL O_2 min^{-1} $g^{-0.67}$) at different activity levels post-frenzy. Error bars represent standard errors and we also report data from earlier studies on hatchling metabolic rates. We denote statistical differences between activity levels within species with *. We converted measurements in previous studies from a mass exponent of 1 to an exponent of 0.67 to correct for allometric relationships between metabolic rate and hatchling mass (Ultsch, 2013).

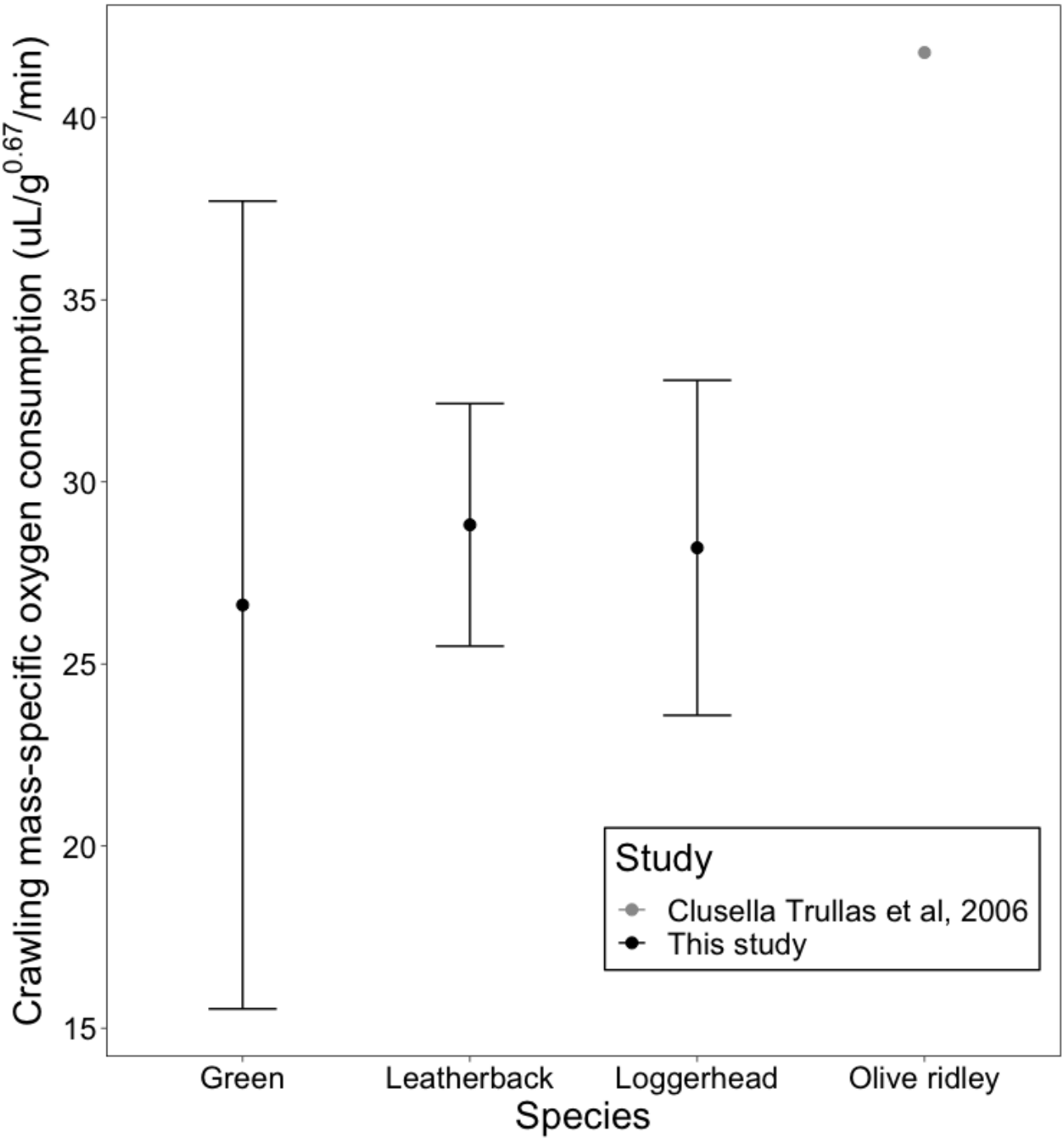

Figure 5.5: Crawling mass-specific metabolic rate (μL O_2 min^{-1} $g^{-0.67}$) of sea turtle hatchlings during the frenzy. Error bars represent standard errors. We also report data from Clusella Trullas *et al.* (2006) who measured metabolic rates in olive ridley hatchlings using doubly-labelled water. We recalculated the olive ridley data point from Clusella Trullas et al. (2006) with a mass exponent of 0.67.

5.4.5 Aerobic scope

Our linear mixed effects model detected differences in aerobic scope among species ($F_{2,383}$ = 49.299, $p<0.0001$), but not among behavioural stages ($F_{1,383}$ = 1.29, $p=0.257$). However, there was also a significant interaction among species and behavioural stage ($F_{1,383}$ = 32.999, $p<0.0001$). Therefore, we used pairwise comparisons to identify significant interactions. During the frenzy, aerobic scope was highest in green hatchlings, lowest in flatback hatchlings and intermediate in olive ridley hatchlings (Supplementary Table 5.6 (p267)). Post-frenzy, flatback hatchling aerobic scopes were higher than olive ridley hatchling aerobic scopes (t_{383}=3.337, $p=0.003$). Flatback aerobic scope was higher post-frenzy than during the frenzy (t_{168}=-5, $p<0.0001$) but olive ridley aerobic scope was higher during the frenzy (t_{177}=3.173, $p=0.002$) (Figure 5.6). We did not include leatherback or loggerhead turtles in our analysis of aerobic scope because we did not measure MMR in these two species. Thus, we cannot determine their maximum ability to increase their metabolic rate above resting.

5.5 DISCUSSION

Our objective was to measure and compare the metabolic rates of five different sea turtle species at different activity levels during the frenzy and post-frenzy behavioural stages. When examining ontogenetic changes in mass-specific metabolic rates, hatchlings that were swimming routinely and maximally generally consumed more oxygen per minute during the frenzy than post-frenzy, although the change from resting oxygen consumption when turtles were quiescent to active, varied among species. Throughout this discussion we refer to mass-specific metabolic rates unless stated otherwise.

5.5.1 Change in oxygen consumption between behavioural stages

5.5.1.1 Resting metabolic rate

Green, olive ridley and loggerhead hatchlings maintained high post-frenzy resting metabolic rates that were similar to those during their respective frenzy rates, while flatback and leatherback hatchlings experienced a decrease in metabolic rate at rest after the frenzy. In our and other studies (Wyneken, 1997; Jones *et al.*, 2007), leatherback hatchlings have shown reductions in metabolic rate during routine swimming, maximal swimming and when at rest post-frenzy compared with the frenzy. Leatherback turtles are entirely pelagic from the time hatchlings leave their natal beaches; they swim continuously during foraging (Davenport, 1987; Eckert, 2002; Salmon *et al.*, 2004) and are not thought to associate with oceanic gyres

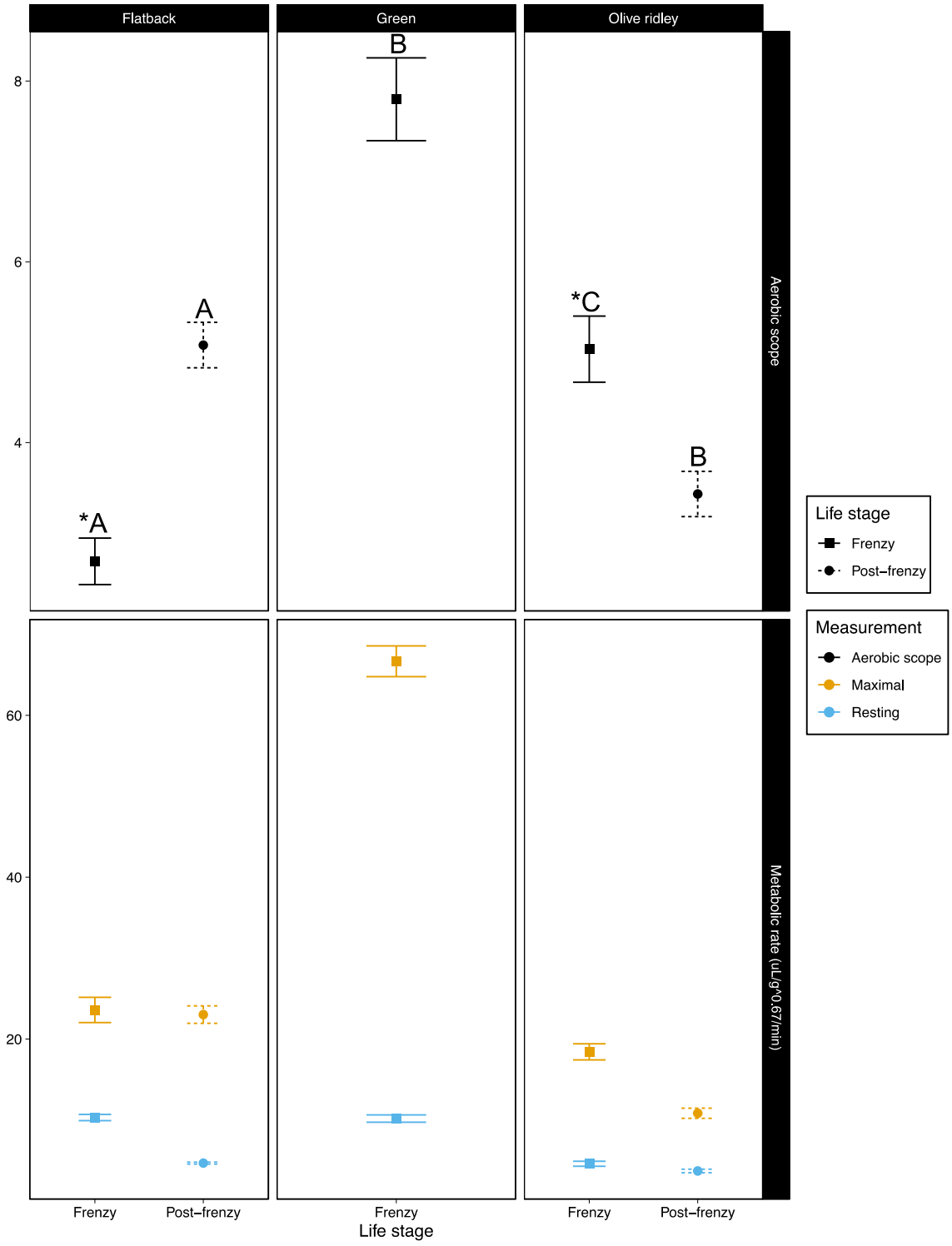

Figure 5.6: Comparison of sea turtle hatchling aerobic scopes during the frenzy and post-frenzy. Error bars represent standard errors. We present aerobic scopes (black) on top and resting (blue) and maximal metabolic rates (yellow) on the bottom. Metabolic rates are reported as μL/$g_{0.67}$/min. Statistical differences between aerobic scopes within species are signified with *. Letters represent statistical similarities between species' aerobic scopes during the frenzy and post-frenzy, respectively.

like other species (Musick & Limpus, 1996). Thus, the reduction in metabolic rate observed in leatherbacks potentially allows conservation of energy when foraging for patchy prey (Lynam *et al.*, 2004; Purcell, 2005; Purcell *et al.*, 2007). In comparison, the reduction in flatback metabolic rate at rest likely reflects their completely neritic life history (Bolten, 2003). Reducing resting metabolic rate allows flatback hatchlings to conserve energy during rest, while experiencing a small decline in maximal metabolic rate allows flatback hatchlings to exert high intensity bursts of energy when escaping predators in neritic waters (Salmon *et al.*, 2009; Pereira *et al.*, 2012). No studies examine the ontogeny of anaerobic scope and capacity in sea turtle hatchlings, although studies on frenzied hatchlings have shown that flatback hatchlings exhibit similar or greater blood lactate concentrations as loggerhead hatchlings (Pereira *et al.*, 2013), despite flatbacks exhibiting less vigorous swimming activity (Pereira *et al.*, 2012). Thus, flatback turtles may utilise anaerobic energy pathways at a greater rate than other sea turtle species.

5.5.1.2 *Metabolic rate during routine and maximal swimming*

All species that we measured had a decrease from frenzy to post-frenzy in either metabolic rate during routine swimming, when hatchlings swam of their own volition, or during maximal swimming, when hatchlings were encouraged to swim maximally by simulating a predation event. This reflects their transition from the frenzy, during which hatchlings attempt to escape predator-dense waters, to the post-frenzy when hatchlings can reduce their activity levels in deeper, less predator-dense pelagic waters (Whelan & Wyneken, 2007). However, while flatback hatchlings do not enter pelagic waters, and instead remain in neritic waters post-frenzy (Bolten, 2003), yet still experience a decrease in metabolic rates post-frenzy. Flatback hatchlings generally perform slow dives when feeding, potentially to more effectively detect and maintain contact with food patches in murky, turbid waters (Salmon *et al.*, 2010). Thus, reduced MMR in post-frenzy flatbacks may reflect this transition from frenzied dispersal to slow diving foraging behaviours. In contrast to our study, Jones *et al.* (2007) found that olive ridley hatchlings had higher maximal metabolic rate post-frenzy than during the frenzy. Olive ridley hatchlings from the Tiwi Islands disperse into the relatively shallow Timor and Arafura seas (Whiting *et al.*, 2007) compared to the eastern Pacific ocean, where the olive ridley hatchlings in the study by Jones *et al.* (2007) disperse. Tiwi Island olive ridleys are therefore likely to experience higher predation rates during dispersal than hatchlings from Costa Rica because shallow waters generally lead to increased predation

rates (Whelan & Wyneken, 2007). Thus, Tiwi Island olive ridley turtles may have experienced selection for higher frenzy maximal metabolic rate to fuel their extended dispersal into deeper waters than Costa Rican olive ridleys. An alternative cause of observed differences in MMR between our study and that of Jones *et al.* (2007) is that 4-week-old olive ridley hatchlings in our study increased in mass by approximately 2g, compared to the 6g increase observed by Jones *et al.* (2007). Two possible explanations are that they were underfed, or their rate of feeding was suppressed in captivity. Another is that olive ridley hatchlings in our study did not feed until approximately 12 days post-emergence. The delayed commencement of feeding in Tiwi Island turtles may have resulted in reduced growth rates, despite Tiwi Island hatchlings initially being heavier (16.46 ± 0.44g) than Costa Rican hatchlings at emergence (13.2 ± 0.08g (Jones *et al.*, 2007)). Thus, the ontogenetic differences in maximal metabolic rate between these two populations may not only reflect genetic, ecological and evolutionary differences but also differences in hatchling quality. The faster growth rates of Costa Rican hatchlings in the Jones *et al.* (2007) study may indicate that those hatchlings were healthier than Tiwi Island olive ridleys. If Tiwi Island olive ridleys were less healthy and of poorer quality, then they may be less capable of reaching or maintaining high MMR.

5.5.2 Comparisons of metabolic rates at different activity levels

Metabolic rate during routine swimming (AMR), crawling (CMR), and rest (RMR) did not always differ. While the difference between AMR and RMR during the frenzy likely reflects the near maximal swimming effort of dispersing sea turtle hatchlings, post-frenzy AMR in loggerheads did not differ from RMR. Post-frenzy, loggerhead hatchlings are thought to be float and wait foragers, similar to olive ridleys (Musick & Limpus, 1996). Thus, a reduction in AMR potentially reflects loggerhead hatchlings becoming relatively inactive and feeding upon surface food items in pelagic waters (Boyle & Limpus, 2008). Leatherback AMR and RMR were also similar during both the frenzy and the post-frenzy. Leatherback hatchlings have a relatively low cost of swimming (Jones *et al.*, 2007) due to their slow, continuous-swimming behaviours. They also grow quickly compared to other sea turtle species (Zug & Parham, 1996; Jones *et al.*, 2011) and the extra energy demands of faster growth may potentially explain higher resting metabolic rates in leatherbacks. Thus, elevated resting

metabolic rate and low metabolic rate during routine swimming led to leatherback hatchlings exhibiting little difference in oxygen consumption at rest and during routine swimming.

Metabolic rate during crawling did not differ from resting metabolic rate during the frenzy except in green hatchlings. Sea turtle hatchlings have been shown to extensively utilise anaerobic energy pathways during the initial stages of the frenzy, including crawling from the nest to the ocean (Dial, 1987; Baldwin *et al.*, 1989; Pereira *et al.*, 2013). Thus, the similar values for resting metabolic rate and crawling metabolic rate may result from the low utilisation of aerobic pathways in favour of anaerobic pathways by crawling hatchlings. Indeed, nesting females have been shown to extensively use anaerobic pathways as they crawl to lay their nests (Jessop & Hamann, 2004). However, aerobic metabolism has been shown to be an important energy pathway for digging and crawling hatchlings (Hamann *et al.*, 2007; Rusli *et al.*, 2016; Pankaew & Milton, 2018). Potentially, hatchlings may utilise anaerobic pathways during bursts of crawling and digging, and then utilise aerobic pathways when removing accumulated lactate during rest periods (Hamann *et al.*, 2007; Pankaew & Milton, 2018), resulting in relatively stable oxygen consumption rates and little lactate accumulation. This potentially explains why Pankaew and Milton (2018) found no difference in plasma lactate concentration of green and loggerhead hatchlings at rest and those that crawled for either 200m or 500m. However, in contrast to our study, Pankaew and Milton (2018) found that oxygen consumption during crawling in both species was higher than in hatchlings at rest. This may reflect the longer crawling trials in their study (>90 min) compared to our study (~40 min) resulting in greater utilisation of aerobic pathways but also the greater accumulation of oxygen debt. Interestingly, they also found that there was no difference in oxygen consumption between hatchlings that swam for 2 hours and those at rest, also different to the results of our study. Potentially, the 'motivation' to crawl or swim among individual hatchlings, clutches and species may vary considerably more than previously thought, resulting in large variation in metabolic measurements and blurred distinctions among activity levels. Thus, similarities among activity levels within studies and differences among studies may be the result of differing levels of 'motivation' among hatchlings. The strength of cues for the hatchlings may also influence hatchling crawling and swimming motivation, while sand characteristics may influence how difficult it is for hatchlings to crawl. Further studies that measure both aerobic and anaerobic metabolism simultaneously

are needed to further elucidate the preferred energetic pathways of hatchlings during dispersal.

5.5.3 Comparisons of metabolic rates among species

5.5.3.1 Resting metabolic rate

Olive ridley resting metabolic rate was consistently lower than that of other species. Olive ridley hatchlings in our study increased in mass by ~2g compared to ~6g by olive ridleys in Jones *et al.* (2007). Thus, the lower metabolic rate at rest in olive ridley hatchlings in our study may have contributed to the slower growth rates of these hatchlings, although the relationship between resting metabolic rate and growth rate is currently unclear (Burton *et al.*, 2011). The lower resting metabolic rate and slower growth rate of our olive ridley hatchlings may also result from differences among populations, or may be a response to other unmeasured variables. In comparison, leatherback hatchlings generally had higher resting metabolic rates than other species during the frenzy and post-frenzy, potentially reflecting their faster growth rates (Zug & Parham, 1996; Jones *et al.*, 2011).

5.5.3.2 Metabolic rate during routine and maximal swimming

Species varied in their oxygen consumption during routine and maximal swimming. However, green sea turtle hatchlings generally had higher metabolic rates during routine swimming (AMR) and maximal swimming (MMR) during the frenzy and post-frenzy compared with other species. These results suggest that green sea turtles expend a greater amount of energy during dispersal compared to other sea turtle species (Pereira *et al.*, 2011; Pereira *et al.*, 2012). Interestingly, loggerhead frenzy AMR was comparable to that of green hatchlings, although loggerhead post-frenzy AMR was lower than greens. Loggerhead and green sea turtles may both exert high levels of energy during the frenzy, but loggerheads appear to switch to less energetically demanding swimming behaviour earlier than green hatchlings. The green and loggerhead hatchlings, tested in our study, that emerge from Floridian beaches are likely to undertake similar dispersal paths along east coast of the mainland USA (Luschi *et al.*, 2003; Putman & Naro-Maciel, 2013; Mansfield *et al.*, 2014). It is possible that loggerhead hatchlings reach their post-hatchling foraging grounds earlier or experience different pelagic habitats to green hatchlings, facilitating an earlier shift to reduced metabolic rates despite following similar dispersal paths. It is unlikely that the size of energy reserves influence metabolic rates because loggerhead hatchlings have been shown to

have larger residual yolk reserves than green hatchlings (Booth & Astill, 2001). In comparison to green and loggerhead hatchlings, leatherback hatchlings exhibited lower AMR compared to other species. Thus, leatherback hatchlings potentially prioritise the duration of time that they can maintain their swimming effort at the expense of the intensity of their swimming effort (Wyneken & Salmon, 1992).

5.5.4 Variation in aerobic scope among species and behavioural stages

We were able to measure both resting and maximal metabolic rates of flatback, green and olive ridley hatchlings. These two measurements represent the aerobic scope, or the capacity of hatchlings to elevate their metabolic rate above maintenance levels (Fry, 1947; Fry & Hart, 1948). Thus, changes in these two measures reflect the physiological limits for hatchlings in terms of their minimum and maximum energy expenditure, although interpretations of aerobic scope should be taken with some caution. Resting and maximal metabolic rates increase with body mass, both within species (Maxwell *et al.*, 2003; Gienger *et al.*, 2017) and among species (Gillooly *et al.*, 2017; White *et al.*, 2019). However, we did not observe a consistent increase in metabolic rate with body mass. This potentially reflects the small range body masses of the hatchlings in our study (range from 16-63g) but also potentially reflects the influence of ontogenetic changes as well as incubation and housing conditions. Similarly, aerobic scopes have generally been shown to increase as body mass increases, both within (Killen *et al.*, 2007) and among species (Bishop, 1999; Weibel *et al.*, 2004). However, like metabolic rates, our study did not observe a consistent increase in aerobic scope with body mass among species. Potentially, this may be the result of ontogenetic changes in our hatchlings resulting in inconsistent changes in aerobic scope, as seen in teleosts (Killen *et al.*, 2007). Thus, we would expect aerobic scopes to increase as our hatchlings continue to grow (Jackson & Prange, 1979; Wyneken, 1997). In comparison to our study, Jones *et al.* (2007) observed an increase in olive ridley aerobic scope over the same life stages as our study. It is possible that factors such as hatchling quality, housing or incubation conditions or population differences may be responsible for this difference. Some authors have suggested that sedentary animals are likely to have higher aerobic scopes because they have lower resting metabolic rates resulting from inactivity and higher maximal metabolic rates because of a greater ability to exert short periods of maximal activity than constantly active individuals (Thompson & Withers, 1997). Conversely, Jackson and Prange (1979) and Weibel *et al.* (2004) proposed that animals with higher aerobic scopes have an increased ability to migrate

because of a greater capacity to increase their energy consumption. However, there is no clear connection between aerobic scopes and migration length or the propensity to migrate (Jones *et al.*, 2007; Southwood & Avens, 2010). Aerobic scopes in fish larvae are narrow, potentially limiting their ability to increase metabolic rate when under physiological stress because of environmental changes (Killen *et al.*, 2007). The ecological relevance of aerobic scopes may also depend on the behaviours and foraging strategies of different taxa. For example, pelagic piscivores may benefit from elevated aerobic scopes because it facilitates a greater increase in metabolic rate when chasing prey. In comparison, benthic ambush predators may also benefit from elevated aerobic scopes because it facilitates faster recovery from burst activity as well as faster digestion of prey during periods of rest (Clark *et al.*, 2013). Thus, the ecological relevance of aerobic scopes may depend on each species' behaviours and remains uncertain overall. In our study, green sea turtles had the highest aerobic scopes during the frenzy, largely because of their extremely high MMR (Figure 5.6). Although flatback hatchlings had higher MMR and RMR than olive ridleys, their aerobic scope was lower than that of olive ridleys. Flatback hatchling mean swim thrust decreases rapidly during the first 24 hours of the frenzy compared to green hatchlings (Pereira *et al.*, 2011; Pereira *et al.*, 2012), supporting the theory of Jackson and Prange (1979) that reduced aerobic scopes may reflect a decreased need to migrate. Thus, it appears that flatback hatchlings may not expend as much energy during dispersal as green or olive ridley hatchlings and that their low aerobic scopes during the frenzy are representative of their shortened migration into neritic waters (Bolten, 2003) compared to pelagic species that undergo longer migrations and have greater aerobic scopes.

5.5.5 Comparing hatchling metabolic rates among studies

Metabolic rates in our study were generally within the range of those reported in previous studies, although not entirely. Oxygen consumption rates in our study were consistently higher than those measured by Prange and Ackerman (1974), Davenport and Oxford (1984) and Lutcavage and Lutz (1986). These differences may have resulted from the methodology and equipment available in those studies, or from differences in genetics, incubation conditions, acclimation conditions, and housing conditions. Lutcavage and Lutz (1986) housed their hatchlings at 20°C and acclimated hatchlings at 24°C before respirometry testing, compared to the warmer temperatures in our study, probably contributing to the higher metabolic rates we measured. Metabolic rates in Clusella Trullas *et al.* (2006)

measured at ~27°C, were consistently higher than those in our study, likely because Clusella Trullas *et al.* (2006) used doubly-labelled water to measure metabolic rates, which may not be a feasible method of determining differences among activity levels, because doubly-labelled water estimates energy consumption over a time period, that may be composed of multiple activities (Jones *et al.*, 2009). Differences in incubation conditions may also explain variation in metabolic rates between our study and others. Most studies on hatchling metabolic rates do not report incubation conditions, despite incubation conditions having been shown to influence metabolic rates in hatchling turtles (O'Steen & Janzen, 1999). Additionally, differences in the time hatchlings were given between pipping the egg and being tested could alter frenzy metabolic rates.

The metabolic rates of hatchlings from Jones *et al.* (2007) were consistently lower than hatchlings in our study during the frenzy. Hatchlings in Jones *et al.* (2007) emerged from natural nests and were allowed to crawl to the ocean before being collected by hand and then tested. Studies that incubate eggs in the laboratory often allow hatchlings to rest in the incubator for 24-48 hours to imitate natural behaviour and yolk utilization. Hatchlings that emerge from the nest and spend time crawling could differ in their oxygen consumption compared to hatchlings that do not undertake these activities. The post-frenzy metabolic rates in our study were not consistently higher or lower than those in Jones et al. (2007), suggesting that differences among studies are unlikely to be the result of differences in methodology, and may instead reflect variation among populations as shown by differences in olive ridley growth rates. Lastly, metabolic rates in Wyneken (1997) were consistently higher than those in our study, although they were closer in value during the frenzy than during the post-frenzy when hatchling metabolic rates in our studies were closer to those in Jones *et al.* (2007). However, the metabolic rates in our study were generally similar to metabolic rates measured in other studies (Figures 5.3 & 5.4), with differences among studies likely reflecting the differences mentioned above. Thus, the metabolic rates measured in our study fall within a similar range to other studies, suggesting that the metabolic rates in our study provide a strong indicator of the energetic demands facing hatchlings during the frenzy and post-frenzy. Differences between our study and other studies likely reflect differences among populations, species, methodology and housing and incubation conditions.

5.5.6 Sea turtle metabolic rates compared with other reptiles.

Sea turtles generally have higher aerobic capacity than other reptile species (Southwood & Avens, 2010; Ultsch, 2013). Resting and standard metabolic rates in hatchling painted turtles, *Chrysemys picta* (0.21 µL O_2 min^{-1} g^{-1}) (Muir *et al.*, 2013) and northern diamondback terrapins *Malaclemys terrapin* (0.58 µL O_2 min^{-1} g^{-1}) (Rowe, 2018) were both lower than the resting metabolic rates of frenzied olive ridley (1.8 µL O_2 min^{-1} g^{-1}) and flatback hatchlings (3.04 µL O_2 min^{-1} g^{-1}) that had the lowest metabolic rates of all sea turtle species measured in our study. Hatchling geckos *Heteronotia binoei* (3.33 µL O_2 min^{-1} g^{-1}) (Andrewartha *et al.*, 2010) and red-eared sliders, *Trachemys scripta elegans* (3.2 µL O_2 min^{-1} g^{-1}) (Eisenreich *et al.*, 2012) had higher resting metabolic rates than olive ridleys and flatbacks but were all lower than loggerhead (3.69 µL O_2 min^{-1} g^{-1}), green (3.7 µL O_2 min^{-1} g^{-1}) and leatherback hatchlings (6.67 µL O_2 min^{-1} g^{-1}) in our study. These species are taxonomically distant from sea turtles and are non-migratory. The closest relative to the Cheloniidae, the common snapping turtle (*Chelydra serpentina*) hatchlings have standard metabolic rates (4.5 µL O_2 min^{-1} g^{-1}) (Eisenreich *et al.*, 2012) that were slightly higher than those of resting loggerheads and greens, yet were considerably less than those of leatherback hatchlings. Sea turtle hatchlings undertake longer dispersal migrations compared to other reptile species, which may explain their elevated metabolic rates. However, metabolic rates alone do not reflect the capacity or proclivity of species to migrate (Southwood & Avens, 2010), and post-frenzy sea turtle resting metabolic rates (1.19-7.31 µL O_2 min^{-1} g^{-1}) remain elevated compared to hatchlings of other reptile species, despite sea turtle hatchlings experiencing a decrease in oxygen consumption during the transition from frenzy to post-frenzy.

Expanding comparisons to include adult reptiles, the desert iguana, *Dipsosaurus dorsalis* (3 µL O_2 min^{-1} g^{-1}) (Bickler & Anderson, 1986)**,** pythons (mean: 0.52 µL O_2 min^{-1} g^{-1}) (Bedford & Christian, 1998) and lizards and snakes (Andrews & Pough, 1985), all had resting metabolic rates that were generally lower than those of sea turtle hatchlings. The vast majority of squamates exhibited resting and standard metabolic rates below 5 µL O_2 min^{-1} g^{-1}, although some exhibited metabolic rates as high 11.67 µL O_2 min^{-1} g^{-1}, which was higher than any of the frenzy or post-frenzy resting metabolic rates measured in our study. However, of the 16 (of 226) published metabolic rates in Andrews and Pough (1985) that were above 5 µL O_2 min^{-1} g^{-1}, eight were recorded in animals that were tested at a temperature of 35°C or above, which may explain the elevated oxygen consumption of these animals compared to

other measurements in the same species. All species measured by Andrews and Pough (1985) were tested within their typical thermal performance range but not necessarily at their thermal performance maximum. When comparing metabolic rates during exercise, sea turtle metabolic rates remained higher than most other species, although varanid lizards and the desert iguana exhibited metabolic rates that are comparable to sea turtle metabolic rates (Bickler & Anderson, 1986; Southwood & Avens, 2010). Thus, the high aerobic capacity of varanid lizards and sea turtles may reflect their active foraging behaviours, rather than reflecting differences in migratory length or frequency (Clemente *et al.*, 2009; Southwood & Avens, 2010). Alternatively, sea turtles can spend up to 86% of their time submerged, generally exhibit short surfacing intervals, rely on aerobic metabolism during dives, have high oxygen storing capacity compared to other reptiles and have low-resistance lungs that facilitate the easy transfer of oxygen from the lungs to the blood (Lapennas & Lutz, 1982; Lutz & Bentley, 1985; Lutcavage & Lutz, 1991; Lutcavage *et al.*, 1992; Southwood *et al.*, 2003; Lutz & Lutcavage, 2017). Thus, the elevated oxygen consumption of sea turtles compared to other reptiles may aid in their ability to quickly replenish oxygen stores between dives. Sea turtles also drastically decrease their heart rates immediately after commencing dives (Southwood *et al.*, 1999) and their activity levels while resting on the sea floor (Reina *et al.*, 2005), to minimise their consumption of oxygen stores whilst submerged. Overall, sea turtle hatchling metabolic rates measured in our study, and in previous studies, are generally higher than those of other reptiles, and the metabolic rates reported in our study represent the considerable aerobic capacity of hatchlings not only during the frenzy, but also post-frenzy.

5.5.7 Conclusions

The mass-specific metabolic rates that we measured here varied by behavioural stage, activity level and species. These differences are largely consistent with ecological and life history differences among species. Leatherback hatchlings exhibited similar metabolic rates during rest and routine swimming, and reduced their metabolic rates as they transitioned from the frenzy to the post-frenzy, possibly reflecting their efficient and continuous swimming behaviours. In contrast, flatback hatchlings exhibited only a small decrease in maximal metabolic rates from the frenzy to the post-frenzy. With their completely neritic life history, maintaining high maximal metabolic rates enables flatback hatchlings to escape predators in predator-dense coastal waters. Olive ridley hatchlings experienced a drop in both resting and maximal metabolic rate post-frenzy, likely reflecting a pelagic float and wait foraging style,

similar to neonate loggerheads. We report comparisons between five of the seven extant species and characterize their early-life metabolic rates. Our results provide the foundations for links between the physiology and ecology of sea turtles, and suggest intriguing next steps towards understanding their environmental and ecological physiology.

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Chapter 6. The ontogeny of sea turtle hatchling swimming performance

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24-week-old green hatchling about to released.

Screenshot taken from a video by Cristina Chang

In press at the *Biological Journal of the Linnean Society*

6.1 ABSTRACT

Sea turtle hatchlings experience high mortality rates during dispersal. To minimise time spent in predator-dense waters, hatchlings typically undergo a period of hyperactivity termed the 'frenzy', characterised by almost continuous swimming for approximately 24 hours. Research has focussed on swimming performance during the frenzy, but our understanding of changes in swimming performance post-frenzy is limited. Thus, we measured green turtle (*Chelonia mydas*) hatchling swimming performance during the frenzy and post-frenzy when the turtles were 4, 12 and 24 weeks old. Using load cells, we recorded thrust production, stroke rates and the time turtles spent performing various swimming gaits. We found that the proportion of time spent powerstroking and thrust generation per powerstroke were the main determinants of overall swimming performance. Older, larger turtles generated more thrust per stroke, but the proportion of time spent powerstroking over the entire swimming trial did not differ among age groups. Hatchlings have been thought to largely utilise currents to reach nursery foraging grounds and our findings suggest that hatchling swimming may also play an important role in directing hatchlings to optimal nursery habitats, supporting recent studies. Additionally, turtle size positively relates to swimming performance in post-frenzy turtles, suggesting that faster-growing turtles may have fitness advantages over slower growing turtles.

6.2 INTRODUCTION

Dispersal from the nesting beach is a vital stage of a sea turtle hatchling's life, as they emerge from the nest, crawl over the sand and swim to deeper waters, all while avoiding numerous predators. Mortality rates vary significantly with water depth and predator density, but can be very high in the first hours after leaving the nest. Hatchlings emerging on beaches with shallower water and higher predator densities can experience predation rates of 30-60% within the first 1-2 h of entering the ocean (Gyuris, 1994; Pilcher *et al.*, 2000), while those from beaches with lower predator densities can experience predation rates as low as 4.6% (Witherington & Salmon, 1992; Stewart & Wyneken, 2004; Whelan & Wyneken, 2007; Duran & Dunbar, 2015). Irrespective of predator density, hatchlings that spend more time in shallow waters experience higher predation rates than those that move out of them sooner (Pilcher *et al.*, 2000; Whelan & Wyneken, 2007).

Sea turtle hatchlings reduce time spent in predator-dense waters by undergoing a period of hyperactivity, characterised by almost continuous swimming for about 24-36 h upon entering the ocean (Wyneken & Salmon, 1992). This period of hyperactivity is termed the 'frenzy', during which time hatchlings spend the majority of their time 'powerstroking', though the duration and intensity of the frenzy differs among species (Chung *et al.*, 2009b; Chung *et al.*, 2009a; Salmon *et al.*, 2009). Powerstroking bouts typically last less than a minute and are characterised by hatchlings swimming with both foreflippers stroking simultaneously in a dorsoventral flapping motion. Between powerstroking bouts, hatchlings also 'dog paddle', a behaviour that consists of 1-5 s bouts when the swimming gait changes to diagonally opposite strokes of the left and right flippers and hind limbs, allowing hatchlings to breathe (Salmon & Wyneken, 1987; Burgess *et al.*, 2006; Booth, 2009).

An overall measure of swimming performance is mean swim thrust i.e., the amount of thrust the hatchling produces during any particular timeframe. During the first 24 h of the frenzy, turtles that have longer powerstroking bouts, spend a greater proportion of the swimming trial powerstroking, stroke at higher frequencies during powerstroking bouts and produce greater mean maximum thrust (i.e., produce more thrust per powerstroke) generally produce greater mean swim thrust (Burgess *et al.*, 2006; Booth, 2009; Booth & Evans, 2011). Hatchlings that produce less thrust are slower swimmers, spend more time in predator dense waters and are more likely to be preyed upon (Gyuris, 1994).

Research on hatchling swimming behaviour has focussed on the first 24 h of dispersal during the frenzy, when predation rates are highest, but less is known about turtle behaviour post-frenzy (Wyneken & Salmon, 1992; Burgess *et al.*, 2006; Booth, 2009). This is largely because turtles are rarely sighted at sea until they are larger juveniles and this gap in their known natural history has been termed the 'lost years' (Carr, 1987). Initial explanations suggested that turtles swim to oceanic currents and passively float to areas of high food availability such as *Sargassum* communities (Carr, 1987; Hays *et al.*, 2010; Shillinger *et al.*, 2012; Witherington *et al.*, 2012). However, more recent studies suggested that turtles may actively swim post-frenzy and select preferable habitats in addition to utilising currents to disperse (Lohmann *et al.*, 2012; Mansfield *et al.*, 2014; Putman & Mansfield, 2015; Briscoe *et al.*, 2016; Gaspar & Lalire, 2017).

These recent studies focused on comparing tracked turtles with models of passively floating particles, but few studies directly investigate the swimming behaviour of turtles post-frenzy. Sea turtles do not feed until ~1 week post-hatching, as they migrate towards foraging grounds (Kraemer & Bennett, 1981). Turtles that do not reach these nutrient-rich foraging grounds are at greater risk of death. Post-frenzy turtles that maintain elevated swimming activity may reach foraging grounds earlier, allowing them to begin feeding sooner or maintain optimal thermal conditions more effectively than slower turtles (Mansfield *et al.*, 2014). This potentially gives them a growth and size advantage over turtles that reach foraging grounds later. Conversely, maintaining elevated swimming activity may deplete yolk reserves more quickly, placing those turtles at greater risk of undernourishment or starvation compared to less active turtles (Kraemer & Bennett, 1981; Jones *et al.*, 2007). Previous studies on the ontogeny of metabolic rates in frenzy and post-frenzy turtles have shown that changes in metabolic rates reflect differences in life history traits between species (Jones *et al.*, 2007; Pereira *et al.*, 2011; Pereira *et al.*, 2012). Thus, understanding how swimming behaviour and activity changes as turtles age could provide greater insight into the differences in life history and dispersal behaviours between species.

To better understand the development of swimming performance in sea turtles and its potential consequences for turtle survival, we investigated turtle swimming attributes from hatching through to post-frenzy swimming at 24 weeks of age. We aimed to identify (1) changes in swimming attributes over time, (2) correlates of morphological differences between turtles on swimming performance and (3) relationships between individual swimming attributes .

6.3 METHODS

6.3.1 Egg collection

Green sea turtle (*Chelonia mydas*) eggs were collected from four nesting females at Capricornia Cays National Park, Heron Island off the coast of Queensland, Australia in January 2017. Eggs (N=75 per clutch) were collected from three clutches and 68 eggs were collected from a fourth clutch. All procedures were approved by the Monash University School of Biological Sciences Animal Ethics Committee (approval BSCI/2016/23). Egg collection and turtle release was conducted under a scientific permit issued by the Queensland Department of Environment and Heritage Protection (WITK177478816). Turtle

housing and experimental procedures were conducted under a research permit issued by the Victorian Department of Environment, Land, Water and Planning (10008208).

6.3.2 Egg transport

Eggs were vacuum-sealed in plastic Ziplock bags with a handpump vacuum (Airlock, Australia) in groups of approximately 20 eggs using the technique of Williamson *et al.* (2017). This process maintains pre-ovipositional arrest and reduces the risks of movement induced mortality because embryos do not develop in the absence of oxygen (Rafferty *et al.*, 2013). Once sealed, the eggs were placed inside insulated containers lined with vermiculite and containing ice packs to maintain the temperature at approximately 12°C. The eggs were transported from Heron Island to Monash University, Clayton, Victoria, where they were placed in incubators and three quarters buried in washed river sand. Time from oviposition to placement in the incubators was approximately 32 h.

6.3.3 Egg incubation

Eggs were incubated at approximately 28°C in groups of 25 eggs per incubator (HovaBator, model 1602N). Eggs were monitored daily for white spot formation, which is the first indicator of active development occurring within the egg (Thompson, 1985). Eggs that showed signs of embryonic death (yellow colour) or fungus were removed from the incubators. The date of hatching (defined as complete emergence from the egg) was recorded for each egg and emerging hatchlings were allowed 48 h to internalise their yolk before locomotor trials commenced.

6.3.4 Turtle morphology

After 48 h, hatchlings were measured. Mass (±0.01 g) was obtained using an electronic balance, while head width, straight carapace length (SCL), straight carapace width (SCW) and flipper length (tip to wrist) measured using digital callipers (±0.01 mm). The same measurements were taken at 4-, 12- and 24-weeks post-hatching.

6.3.5 Measuring turtle swimming performance

Turtles were fitted with elasticised fabric harnesses that did not inhibit flipper movement. The harness was attached via monofilament fishing line (length: 35cm) to a 5N load cell (PS-

2201, Pasco, USA) connected to a load cell amplifier (PS-2198, Pasco, USA) programmed to sample 20 times per second (Figure 6.1). Before each trial, the load cell was calibrated by hanging a known mass from it. Turtles swam in glass tanks with a white light at one end to induce unidirectional swimming in water maintained at approximately 27± 0.4°C with an aquarium heater and monitored with an electronic thermometer.

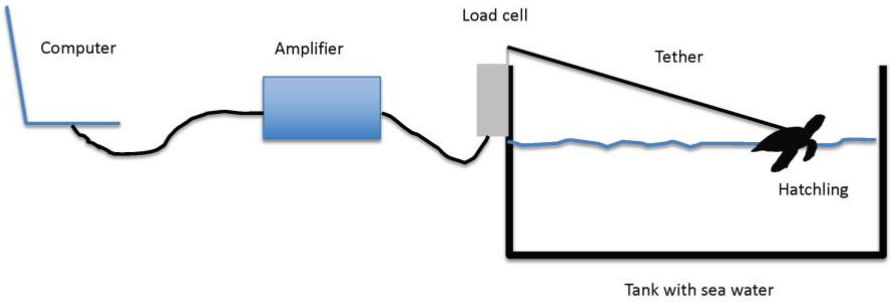

Figure 6.1: The set-up used to test swimming performance in this study.

Hatchlings were allowed to swim for 2 h when tested at 0 weeks of age (n = 60) before being removed from the harness and placed in their housing tanks (described below). Turtle swimming performance was measured again for 30 min in the same manner at 4 (n = 60), 12 (n = 12) and 24 weeks of age (n = 12). Turtles were tested in a darkened room during daylight hours.

These methods enable the quantification of five swimming attributes; (1) mean swimming thrust (Newtons, N) or the mean thrust produced by a turtle during its entire swimming trial. *Mean Swim Thrust* is an overall measure of swimming performance because it incorporates all swimming attributes into a single value. (2) *Proportion of time spent powerstroking* provides a measure of activity, expressed as a percentage of total time spent swimming using the powerstroke gait. (3) *Mean Maximum Thrust* per powerstroke bout (N), measures the average peak thrust produced by each powerstroke. (4) Duration of powerstroking bouts (s) is the time from the start to the end of a powerstroking bout (*Powerstroke Bout Duration*) and (5) *Powerstroke Frequency*, as powerstrokes per min, gives a measure of the rate of flapping within powerstroking bouts (Burgess *et al.*, 2006; Booth, 2009).

6.3.6 Animal housing and release

Turtles were housed in 3L and 10L plastic tanks or in larger glass tanks separated with egg crating (12.5mm grid, Aquasonic, Australia). Tanks were kept clean by a continuous flow-through system consisting of a drum filter (Faivre 60 series, Faivre, France), fluid sand bed filters (RK2 systems, USA), a protein skimmer (RK10AC, RK2 systems, USA), a UV filter (240W UV steriliser, Emperor Aquatics, USA) and an ozone steriliser (RK300MG, RK2 systems, USA). Water quality was monitored daily using OxyGuard hand-held monitors (Technolab, Australia). Water temperature was maintained at 26-27°C using a heater (3kW heater, Shego, Germany) and a chiller (FBT175SSD, Toyesi, Australia). Animals were maintained under a day/night cycle of 12/12 h and provided with UV lighting (Exo Terra Repti Glo 5.0 25W). Turtles were fed daily with commercial turtle pellets (4mm Marine float range, Ridley Aquafeed).

At the conclusion of experiments turtles were transported in plastic crates lined with foam back to Heron Island for release, where they were released into the East Australian Current.

6.3.7 Statistical analysis

The following statistical analyses were conducted in R (R Core Team, 2014) using the lme4 library (Bates, 2007) and the lmerTest package (Kuznetsova *et al.*, 2017). Statistical differences between age groups were determined Tukey's HSD in the emmeans package (Lenth *et al.*, 2018). R^2 values were obtained from linear regressions of the fixed effects. For comparisons of swimming attributes and morphology among age groups, we obtained values of Cohen's D in the effsize package (Torchiano, 2020).

The change in turtle morphology and in swimming attributes were analysed with age as the fixed effect and clutch and hatchling ID as the random effects to account for repeated measures. Models that incorporated proportion of time spent powerstroking were used with binomial probability distributions and cloglog link functions because the proportion of time spent powerstroking was negatively skewed.

Relationships between morphology and swimming attributes and between two different swimming attributes were analysed with swimming attributes as the response variable and either morphology or swimming attributes as the fixed effect. Clutch and hatchling identity were random effects to account for repeated measures. To minimise errors associated with

multiple comparisons, we focused on biologically significant relationships e.g., between flipper length and mean maximum thrust.

6.4 RESULTS

6.4.1 Incubation duration and hatching success

Mean incubation duration among the 12 incubators was 66.17 ± 2.52 d (N=293 eggs, N=4 clutches, range: 61-70 days) and mean hatching success among the 12 incubators was 92.47 ± 5.24% (range: 86.36-100%).

6.4.2 Turtle morphology

Mass ($F_{1,140.2}$=1096, p<0.001), SCL ($F_{1,140.8}$=1066.8, p<0.001), SCW ($F_{1,140.1}$=779.45, p<0.001) and head width ($F_{1,139.9}$=815.74, p<0.001) increased at all weeks of age (Table 6.1). Flipper length also increased as hatchlings aged ($F_{1,142}$=584.68, p<0.001) but flipper length did not increase from 12 to 24 weeks of age ($t_{135.4}$=-1.49, p=0.44). Results of Tukey's HSD tests among age groups can be found in Table 6.1 (p269).

6.4.3 Swimming performance

Sea turtle swimming attributes changed significantly as the turtles aged (Table 6.2). Mean maximum thrust and mean swim thrust both increased each week from 0 to 12 wk of age (Figure 6.2). There was no change between 12 and 24 wks. The age of the turtles had a significant effect on powerstroke bout duration and powerstroke frequency but the change in these swimming attributes varied between ages (Figure 6.2). Powerstroke bout duration was longer at 4 weeks old compared to 12 and 24 wk old, with a difference in bout duration of about 1 s. Powerstroke bout duration at 0 weeks of age did not differ from any other age group. Powerstroke frequency was similar at 0, 12 and 24 weeks of age but 4-week-old turtles had significantly lower powerstroke frequencies than the other age groups (Figure 6.2). Proportion of time powerstroking did not change as the hatchlings aged (Figure 6.2). Comparisons of the performance metrics with age by Tukey's HSD tests are summarised in Supplementary Table 6.2 (p270).

Table 6.1: Turtle morphological measurements from hatching to 24 weeks of age. Data are presented as mean ± SD (range).

	Mass (g)	Straight carapace length (mm)	Straight carapace width (mm)	Front flipper length (mm)	Head width (mm)
Week 0	26.31 ± 3.09 (20.89-31.32)	51.48 ± 2.25 (47.21-55.59)	40.27 ± 2.45 (28.83-44.29)	44.91 ± 2.19 (39.66-49.21)	15.69 ± 0.57 (14.51-16.59)
Week 4	42.10 ± 5.01 (32.36-52.05)	62.02 ± 2.65 (56.14-68.47)	52.58 ± 3.02 (46.49-59.6)	51.02 ± 2.13 (47.67-57.33)	17.23 ± 0.53 (16.07-18.32)
Week 12	108.51 ± 8.54 (89.83-119.67)	88.35 ± 2.80 (83.65-92.02)	77.92 ± 3.30 (73.96-82.85)	65.14 ± 2.08 (62.61-68.19)	21.21 ± 0.67 (20.16-22.36)
Week 24	120.28 ± 11.86 (92.3-132.7)	93.79 ± 3.59 (86.7-99.3)	81.14 ± 4.59 (72.75-86.24)	66.77 ± 3.74 (61.71-75.01)	22.00 ± 0.69 (20.29-22.56)

6.4.4 Swimming attributes are highly related

Individual attributes of swimming performance had strong influences on other attributes among age groups. Mean swim thrust increased with proportion of time powerstroking ($z=7.17$, $p<0.001$, $R^2=0.161$) and with mean maximum thrust ($F_{1,139.9}=265.85$, $p<0.001$, $R^2=0.65$).

Proportion of time powerstroking had a positive relationship with mean maximum thrust ($z=6.95$, $p<0.001$, $R^2=0.48$).

Longer powerstroke bout duration also resulted in higher proportion of time spent powerstroking ($z=3.09$, $p=0.002$, $R^2=0.009$), though the relationship was weak.

Powerstroke frequency had a negative relationship with proportion of time powerstroking ($z=-4.78$, $p<0.001$, $R^2=0.23$) and with powerstroke bout duration ($F_{1,107.5}=32.26$, $p<0.001$, $R^2=0.16$) so that both increased as stroke rate during a powerstroking bout decreased.

6.4.5 Morphology had a strong effect on swimming performance

Turtle flipper length and mass had a strong influence on swimming performance with older, larger turtles generally producing more thrust between and within age groups. Among all age groups, mean swim thrust increased as flipper length increased ($F_{1,140.3}=100.01$, $p<0.001$, $R^2=0.39$) as did mean maximum thrust ($F_{1,136.7}=416.8$, $p<0.001$, $R^2=0.73$). Among age groups, heavier turtles produced greater mean swim thrust ($F_{1,140.4}=133.8$, $p<0.001$, $R^2=0.48$) and greater mean maximum thrust ($F_{1,140.3}=617.6$, $p<0.001$, $R^2=0.81$).

Table 6.2: Turtle swimming attributes from hatching to 24 weeks of age. Data for each age group are presented as mean ± SD. We also report the results of linear mixed effects models on the change in each locomotor performance variable over time.

	Mean swim thrust (N)	**Mean maximum thrust (N)**	**Time spent powerstroking (%)**	**Duration of powerstroking bouts (s)**	**Stroke-rate during powerstroking bouts (str/min)**
Week 0	0.0332 ± 0.015 (0.0053-0.0688)	0.1234 ± 0.037 (0.0322-0.1803)	53.8 ± 23.1 (7.4-88.1)	4.65 ± 2.43 (1.92-14.55)	176.1 ± 21.4 (116.6-226)
Week 4	0.0536 ± 0.011 (0.0168-0.0773)	0.2683 ± 0.044 (0.1854-0.3542)	70.4 ± 13.8 (28.2-90.2)	5.34 ± 1.83 (2.78-12.18)	147.2 ± 14.5 (117-191.8)
Week 12	0.0937 ± 0.045 (0.0241-0.1434)	0.5295 ± 0.087 (0.4394-0.7165)	44.8 ± 20.3 (6.9-64.7)	2.85 ± 0.81 (1.55-4.2)	185.9 ± 44.8 (122.3-245)
Week 24	0.1032 ± 0.066 (0.0048-0.189)	0.5506 ± 0.173 (0.2769-0.782)	49.1 ± 30.7 (2.6-81.3)	3.36 ± 1.74 (0.9-6.16)	199 ±54.9 (131.2-333.3)
Change in performance over time	$F_{1,139.9}$=102.65, p<0.001	$F_{1,140.2}$=362.8, p<0.001	z=1.33, p=0.19	$F_{1,134.9}$=6.89, p=0.01	$F_{1,140.4}$=8.53, p=0.004

6.5 DISCUSSION

Swimming velocity in animals is largely determined by the amount of thrust generated by individuals and the amount of drag that they need to overcome (Prange, 1976). Thrust is generated by turtles using the flippers and acts to move the turtle forward, while drag is the resistance due to the water's viscosity and the surface area of the turtle as the turtle moves through it. As a sea turtle generates more thrust (i.e., its swimming performance increases), its speed also increases but as drag increases, the turtle slows down or requires more thrust to start moving if stationary (Watson & Granger, 1998; Jones *et al.*, 2011; Jones *et al.*, 2013). In our study, we measured the amount of thrust that each turtle produced per stroke and during the entire swimming trial. We did not quantify drag and therefore could not calculate exact swimming speeds. However, the amount of drag that the turtles produced in each age group is likely to be similar because the turtles remained a similar shape from hatching to 24 weeks of age. For example, an adult leatherback turtle that had ~5.8 times the frontal area (m^2)

and was ~2 times longer and wider than a juvenile leatherback, only had 1.27 times the drag coefficient of the smaller juvenile (Jones *et al.*, 2011).Thus, measuring thrust production provides a strong measure of the relative swimming speed of each turtle. Considering that thrust is the main determinant of turtle swimming speeds, an overall measure of swimming performance is mean swim thrust (Burgess *et al.*, 2006; Booth, 2009). This measure integrates each attribute of swimming performance into a single value. Sea turtles increase their swimming performance in two main ways; producing more thrust per stroke and

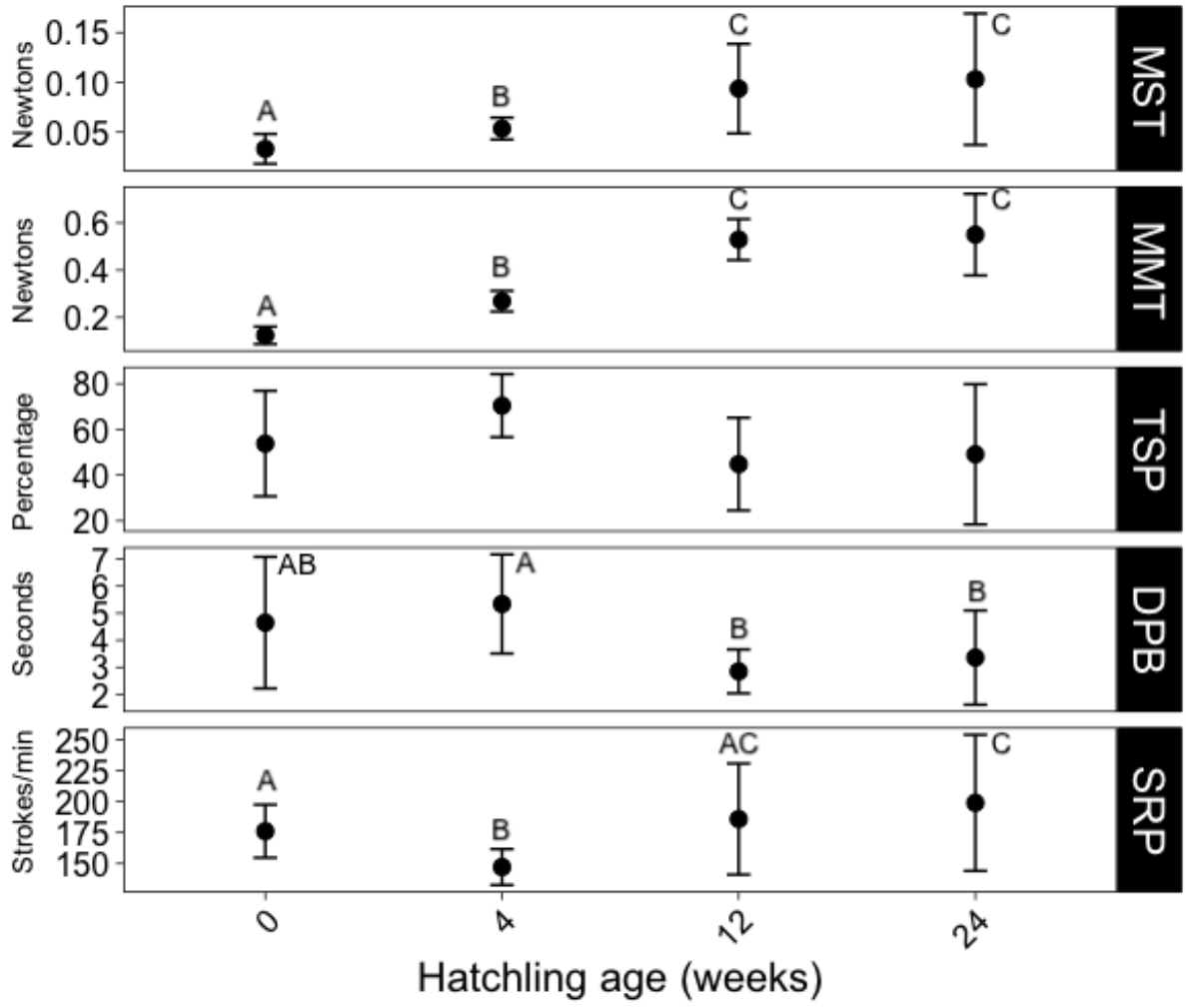

Figure 6.2: Change in swimming attributes from hatching to 24 weeks of age. We present the data as mean ± standard deviation. Different letters refer to age groups that significantly differed based on Tukey's HSD test. MST=Mean swimming thrust (N), MMT=Mean maximum thrust (N), TSP=Time spent powerstroking (%), DPB=Duration of powerstroking bouts (s) and SRP=Stroke-rates during powerstroking bouts (strokes per min).

completing more powerstrokes by spending more time powerstroking or increasing powerstroke frequency.

6.5.1 Changes in swimming attributes over time

When examining changes in swimming attributes over time, we expected that a turtle's motivation to swim would decrease as it aged. This was based on previously observed changes in swimming behaviour within the first 24 hours of the 'frenzy' (Burgess *et al.*, 2006; Booth, 2009; Ischer *et al.*, 2009) and theoretically might occur when turtles enter oceanic currents and passively disperse (Carr, 1987). However, the proportion of time spent powerstroking, a key attribute of swimming performance, did not change from hatching to 24 weeks old (Figure 6.2), despite decreasing when measured continuously during the first 24 hours in the water (Booth, 2009). Additionally, hatchlings also decreased their nocturnal activity post-frenzy (Wyneken & Salmon, 1992; Salmon *et al.*, 2009). It is possible that the continuous exertion of the 'frenzy' leads to decreased proportion of time spent powerstroking via depletion of muscle glycogen (Hill *et al.*, 2004) or accumulation of blood lactate (Baldwin *et al.*, 1989; Pereira *et al.*, 2013). The restoration of glycogen levels or the removal of lactate during extended rest periods may allow turtles to regain their ability to powerstroke for longer periods. If swimming tests had been conducted for 24 hours rather than 2 hours in our study, it is likely that we would have observed decreases in the proportion of time spent powerstroking as hatchlings aged. Consequently, we were unable to determine whether older turtles reduce the proportion of time they spend powerstroking earlier than 'frenzy' hatchlings but over the time periods measured here, sea turtle swimming activity remained relatively constant. Alternatively, the lack of orientation cues such as waves and magnetic fields may result in hatchlings maintaining activity levels similar to frenzy levels even at 4 weeks of age as they attempt to reach foraging grounds (Salmon & Wyneken, 1987; Salmon & Lohmann, 1989; Lohmann, 1991). Overall, sea turtles remain motivated to swim by a light stimulus even at 24 weeks of age.

Though the proportion of time spent powerstroking over their entire swimming trial did not change as hatchlings grew, powerstroking bout durations were longest in 4-week-old turtles, intermediate in frenzy hatchlings and shortest in 12 and 24-week-old hatchlings. Powerstroke frequencies were lowest in 4-week-old hatchlings and then increased as hatchlings aged (Figure 6.2). Potentially, stroke rates are initially high during the frenzy to

increase hatchling swimming speed in neritic waters (Wyneken & Salmon, 1992; Gyuris, 1994). At 4-weeks-old, turtles experienced decreased stroke rates even though powerstroking bout durations were similar between frenzy and 4-week-old hatchlings. Reduced powerstroke frequencies at 4 weeks of age may allow turtles to maintain powerstroking bout durations similar to the frenzy but with lower energetic costs. Finally, 12- and 24-week-old turtles switch to short burst, high intensity swimming behaviour that may enable them to catch prey and avoid predation at foraging grounds. However, these changes largely negate each other because increases in powerstroke frequencies appear to necessitate reductions in powerstroking bout durations and vice-versa. Among sea turtle species, powerstroke frequency has been shown to both increase and decrease ontogenetically (Jones *et al.*, 2007; Gatto & Reina, In press). Thus, these changes appear to reflect life history differences among species rather than the effects of altered morphometrics because of increased flipper length (Stevens *et al.*, 2018). Overall, based on the proportion of time that turtles spent powerstroking, the duration of powerstroking bouts and powerstroke frequencies, it does not appear that a turtle's motivation to swim changes significantly over the first 24 weeks of life. Although statistically significant, differences between age groups are very small and relationships are weak, suggesting little biologically significance.

Though a turtle's motivation to swim may not change, swimming speedsas indicated by mean swim thrust, increased as the turtles grew older. Mean swim thrust increased at all ages, as did mean maximum thrust per powerstroking bout. It is very likely that the greater size and strength of older turtles, as well as their larger flippers, resulted in the increased mean maximum thrust, which in turn resulted in increased mean swim thrust production. Older, larger turtles are likely to be faster swimmers than smaller, younger turtles because of their ability to produce more thrust when powerstroking rather than through changes in swimming behaviour. The consistency of the swimming attributes proportion of time spent powerstroking, powerstroke bout durations and powerstroke frequencies as turtles age suggests that turtles remain relatively active post-frenzy.

6.5.2 Comparison of swimming performance among studies

Making direct comparisons between studies with different designs can be difficult. Measures of swimming performance can differ between studies because of variation in incubation and housing conditions, surrounding stimuli such as light and differences in the angle of the

monofilament line used to connect the hatchling to the load cell (Salmon & Wyneken, 1987; Burgess *et al.*, 2006; Delmas *et al.*, 2007). However, we can compare the time spent powerstroking and stroke rates during powerstroking bouts at the same temperature with greater confidence than measures of thrust production because these attributes are less reliant on methods for measurement of thrust production. Although turtles in our study powerstroked at higher frequencies than turtles from Booth (2009), they spent less time powerstroking. The increased energy requirements of powerstroking at faster rates may have resulted in turtles in our study requiring longer breaks from powerstroking, resulting in a smaller proportion of time spent powerstroking overall. Turtles from our study also powerstroked at higher frequencies than turtles from Burgess *et al.* (2006). However, Burgess *et al.* (2006) reported significant variation in the proportion of time that turtles spent powerstroking (25-70%) with turtles in our study falling within this range (Table 6.2). It is likely that the variation in turtle swimming performance seen between these studies is a reflection of maternal variation and differences in incubation conditions, experimental force-measuring equipment and each turtles' motivation to swim (Booth *et al.*, 2004; Burgess *et al.*, 2006; Booth, 2017).

6.5.3 Ecological implications

Turtles that produce less thrust and swim more slowly than other turtles are at greater risk of predation and mortality for a number of reasons. Initially during dispersal, hatchlings swim in coastal waters that are often predator dense. Slower swimming hatchlings will spend more time in these waters and are therefore at greater risk of predation (Gyuris, 1994). Additionally, slower, weaker swimmers are less likely to be able to swim past waves, actively select preferred habitats or maintain contact with reliable food sources (Putman *et al.*, 2012; Cavallo *et al.*, 2015). Feeding earlier and remaining in optimal habitats potentially provides turtles with short- and long-term advantages in growth rates and reproductive output compared to turtles that take longer to reach foraging grounds (Ebenman, 1988; Janzen, 1993; Chaloupka *et al.*, 2004). Larger turtles experience reduced predation rates because predators become gape-limited (Persson *et al.*, 1996; Gyuris, 2000; Salmon & Scholl, 2014; Stevens *et al.*, 2018) and generally are able to generate more thrust per powerstroke compared to smaller turtles. Consequently, turtles that are more forceful swimmers may experience numerous benefits during and post-dispersal compared to slower swimmers.

The initial 'frenzy' remains the most significant period for hatchlings during dispersal, mainly due to increased predation risk, but it remains important to consider the impact of variation in swimming performance over longer time periods. Initial theories on the dispersal of sea turtle hatchlings suggested that after the 'frenzy' hatchlings passively floated with currents that carried them to post-hatchling feeding grounds (Carr, 1987), where hatchlings grow in size. However, recent studies have suggested that hatchlings may not passively disperse as first thought (Lohmann *et al.*, 2012; Mansfield *et al.*, 2014; Putman & Mansfield, 2015; Briscoe *et al.*, 2016) and that instead, they may actively select habitats for their food availability, protection or thermal suitability (Mansfield *et al.*, 2014). If hatchlings passively disperse, then it is likely that their motivation to swim would significantly decrease post-frenzy. However, we showed that turtles up to 24 weeks of age maintain powerstroke frequencies, powerstroke bout durations and the proportion of time spent powerstroking at levels comparable to the initial 'frenzy', while simultaneously increasing their thrust production. This suggests that turtles retain a considerable motivation to swim for nearly six months post-hatching and remain quite active even when utilising currents to reach feeding grounds. However, turtles also use a number of other cues to orientate themselves during dispersal including light, magnetic cues and waves (Salmon & Wyneken, 1987; Salmon & Lohmann, 1989; Wyneken & Salmon, 1992; Tuxbury & Salmon, 2005; Lohmann *et al.*, 2012) and it is possible that turtles in our study maintained their swimming effort because they were not sensing location changes. It is unknown whether turtles maintain constant swimming effort until they reach preferred habitats or whether they adjust their swimming effort to minimise metabolic costs. Overall, the swimming behaviours measured here and the dispersal behaviour of tracked hatchlings in natural settings (Mansfield *et al.*, 2014; Putman & Mansfield, 2015; Briscoe *et al.*, 2016) indicate that hatchlings likely have the potential to actively select preferable habitats and therefore are not completely subject to ocean currents when dispersing.

Future research into the dispersal of sea turtle hatchlings will need to consider how and why hatchlings select certain habitats in conjunction with how changes to currents will impact their dispersal. This will allow the identification and protection of preferable habitats associated with key dispersal currents. Additionally, the ontogeny of turtle swimming performance may differ in oceanic waters as turtles alter their behaviour in response to various cues (Salmon & Wyneken, 1987; Salmon & Lohmann, 1989; Tuxbury & Salmon,

2005; Lohmann *et al.*, 2012) or if turtles adjust their swimming effort to minimise metabolic costs. Finally, hatchling frenzy behaviour has been shown to vary between species, potentially reflecting life history differences (Chung *et al.*, 2009a; Salmon *et al.*, 2009). Further research is required to investigate whether post-frenzy behaviours also match this life history variation between species.

6.5.4 Conclusions

In our study, green sea turtles exhibited increased swimming performance, as indicated by mean swim thrust, as they grew older. This increase in performance was largely driven by increases in mean maximum thrust production. In effect, as turtles grew larger, they were able to generate more thrust per stroke and thus, were able to generate more mean swim thrust. In comparison, other swimming attributes such as stroke rate frequency during powerstroking bouts, proportion of time spent powerstroking and duration of powerstroking bouts did not change as the turtles grew. Our findings support recent studies that suggest that turtles remain active swimmers as they disperse post-frenzy and actively select optimal habitats for thermal suitability or food availability (Mansfield *et al.*, 2014; Putman & Mansfield, 2015; Briscoe *et al.*, 2016).

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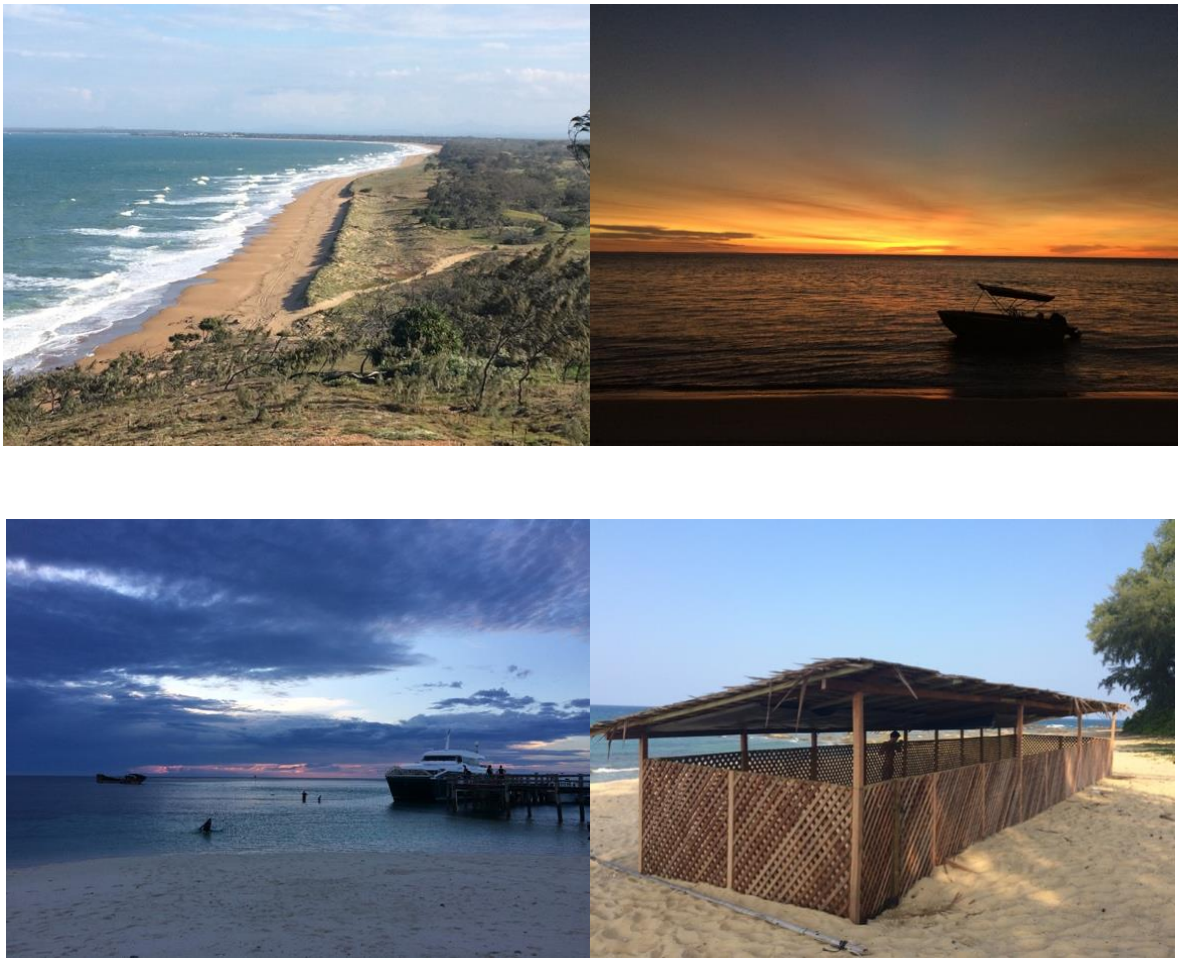
Chapter 7. General Discussion



Curtis Island (flatbacks), the Tiwi Islands (ridleys), Heron Island (greens) and the Lang Tengah Turtle Watch hatchery in Terengganu, Malaysia.

Photos taken by Christopher Gatto

7.1 SUMMARY OF FINDINGS

In this study, I measured the response of sea turtle hatchling locomotor performance and thermal tolerance to various moisture levels during incubation. Additionally, I measured the ontogenetic change in locomotor performance and metabolic rates in multiple sea turtle species. These results broaden our understanding of how incubation conditions influence hatchling dispersal ability and thus, survival rates. By also measuring ontogenetic changes in dispersal ability, I provided insight into how incubation conditions may affect hatchling recruitment and population dynamics over a hatchling's entire dispersal, rather than just projecting potential implications based on initial hatchling traits. Lastly, I highlighted contrasts in dispersal ability among species and related these differences to variation in life history among species. This provided further insight into how incubation conditions may impact species differently. The following sections summarise the main findings of each chapter, discuss the overall ecological implications and consider potential limitations and future directions. I summarise my aims, specific chapter questions and key findings for each chapter in Figure 7.1.

7.1.1 A review of incubation conditions and their effects on hatchling phenotypes in the Reptilia (Chapter 2)

Research on the effects of incubation conditions on hatchling traits in oviparous species has extensively focused on incubation temperatures and its effect on primary sex ratios, hatchling morphology and hatching success. Studies have recently begun to investigate the effects of incubation temperatures on hatchling locomotor performance (Burgess *et al.*, 2006; Booth, 2017; Booth, 2018), although studies on environmental variables other than temperature are less common. In chapter 2, I reviewed how temperature, moisture, salinity and oxygen concentration influence developmental success and phenotypes in a wide range of oviparous reptilian species and identify current gaps in the literature. I also discussed how environmental factors interact to determine phenotypes and assess the potential consequences of altered incubation conditions for adult populations. Among environmental factors, most studies have focused on turtles and lizards and generally, they have focused on isolated environmental factors, with few studies incorporating two or more interacting variables. Future studies should consider examining the effects of multiple, interacting environmental effects in order to create a broader understanding of how incubation conditions in natural nests are influencing embryonic development and hatchling traits in oviparous reptiles.

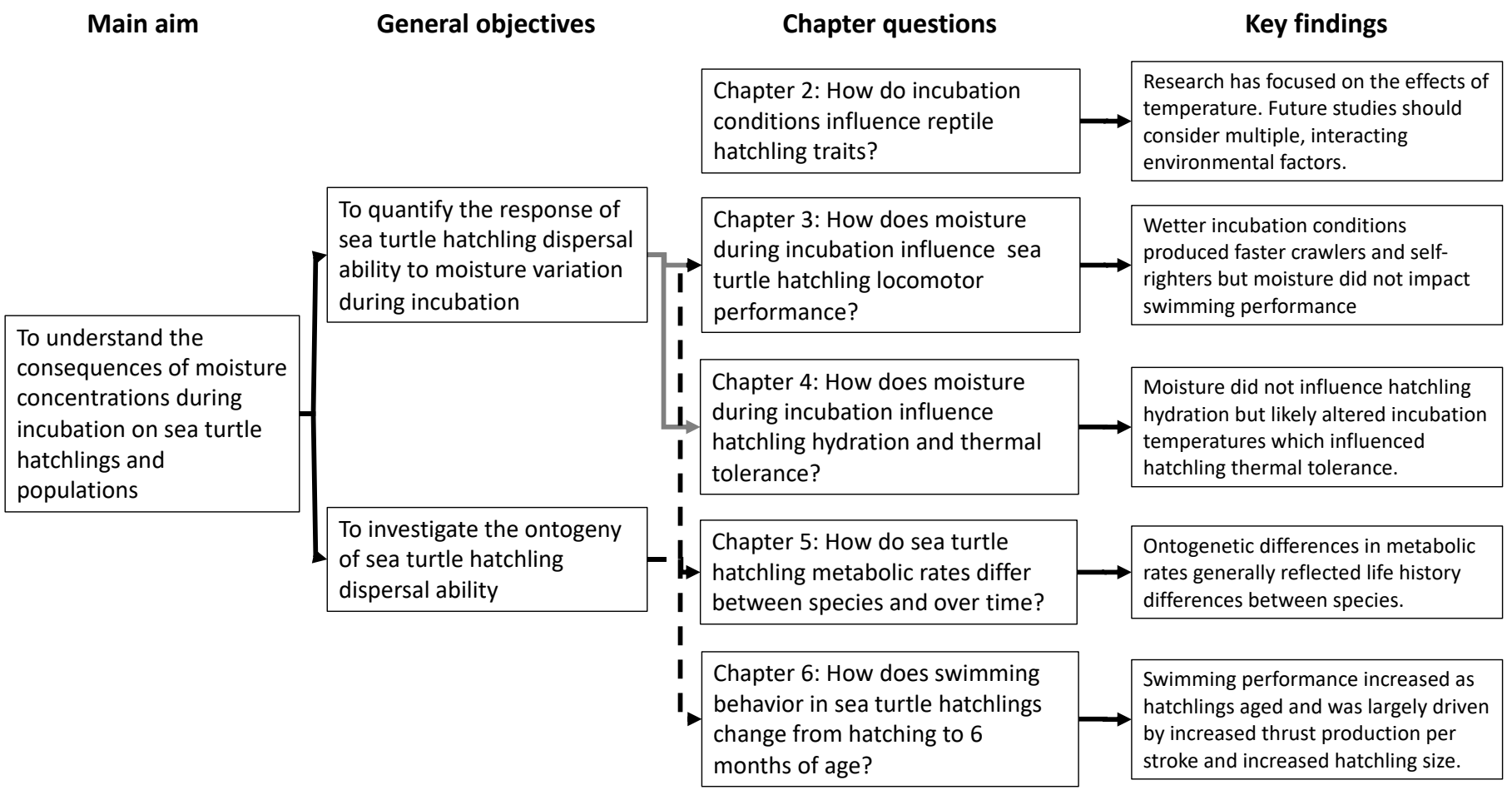


Figure 7.1: Main research aims, general thesis structure and key findings.

7.1.2 Sea turtle hatchling locomotor performance: incubation moisture effects, ontogeny and species-specific patterns (Chapter 3)

Hatchling survival during dispersal is largely determined by predator density in nearshore waters and how long hatchlings spend in those predator dense waters (Gyuris, 1994; Duran & Dunbar, 2015). Thus, sea turtle hatchling swimming behaviours and locomotor performance determine hatchling survival rates by influencing how much time hatchlings spend in predator-dense waters, their ability to combat waves and currents and how long they take to reach foraging grounds (Booth, 2009; Putman *et al.*, 2012). I measured the response of sea turtle hatchling self-righting ability, crawling and swimming performance to various moisture levels during incubation in chapter 3. I also evaluated the ontogeny of each species' locomotor performance and discussed the consequences of moisture concentrations during incubation for population dynamics and viability. Hatchlings incubated in dry conditions were slower crawlers and took longer to self-right than hatchlings from wet nests, but moisture had no influence on hatchling swimming behaviours or overall swimming performance. I hypothesise that hatchlings from dry nests may emerge more dehydrated than hatchlings from wet nests, and thus, are slower crawlers and take longer to self-right. Once hatchlings enter the ocean, they consume large quantities of water and rehydrate (Reina *et al.*, 2002). Therefore, the now hydrated hatchlings do not differ in their swimming performance compared to hatchlings from wet nests. Moisture levels during incubation are likely to influence hatchling survival as hatchlings crawl to the ocean, while the effect on hatchling dispersal after they enter the water is likely to be minimal. Changes in precipitation may have a greater impact on turtles that nest on beaches with high levels of terrestrial predation.

7.1.3 The role of incubation environment in determining sea turtle hatchling thermal tolerance (Chapter 4)

As air, sand and ocean temperatures all rise, the ability of sea turtle hatchlings to tolerate extreme temperatures will play a vital role in determining their ability to survive dispersal. In chapter 4, I tested whether incubation conditions, specifically moisture concentrations, influenced sea turtle hatchling thermal tolerance. Further, I measured hatchling packed cell volume and total protein to see whether moisture concentration during incubation influenced thermal tolerance via hatchling hydration. Moisture level during incubation did not influence hatchling hydration or thermal tolerance. However, using incubation duration as a proxy for incubation temperature, dry nests were considerably warmer than wet nests and those

hatchlings from warmer nests had significantly higher thermal tolerance than hatchlings from cool nests. Watering nests has been proposed as one method for combatting rising sand and nest temperatures under climate change (Hill *et al.*, 2015). Watering nests may reduce incubation temperatures, promoting successful embryonic development, however may simultaneously reduce hatchling thermal tolerance, decreasing hatchling recruitment into adult populations. Conversely, hatchlings that survive incubation in warm nests may have an increased ability to survive those warm temperatures during dispersal.

7.1.4 Ontogeny and ecological significance of metabolic rates in sea turtle hatchlings (Chapter 5)

The 'frenzy' is most intense during the first 24 hours of dispersal, but hatchlings continue to swim towards feeding grounds for days post-frenzy (Wyneken & Salmon, 1992). Initial studies on the ontogeny of metabolic rates in sea turtle hatchlings have suggested that differences among species largely reflect variation in life history (Jones *et al.*, 2007). Thus, similar changes in metabolic rates may have different consequences for species depending on their life history (e.g. the completely neritic life history of flatbacks compared to the pelagic life history of green turtles). I compared the ontogenetic changes of five sea turtle species, providing a comprehensive comparison of hatchling metabolic rates in multiple species. As suggested in previous studies, the ontogeny of hatchling metabolic rates reflected differences in life history. For example, leatherback hatchlings experienced a reduction in metabolic rate at rest and during routine swimming from the frenzy to post-frenzy, while flatback hatchlings experienced the same decrease in resting metabolic rate. The shared decrease in metabolic rate during routine and maximal swimming likely reflects the fact that all species experience the frenzy and need to disperse from nesting beaches as quickly as possible. However, once in pelagic waters, leatherbacks swim continuously during foraging (Davenport, 1987; Eckert, 2002), and a reduction in metabolic rate likely reflects their efficient, continuous foraging behaviours. Comparatively, flatback hatchlings experienced a much smaller decrease in maximal metabolic rate, which may aid flatback hatchlings that remain in neritic waters and must exert high intensity swimming efforts to escape predation. In that chapter, I was also able to identify differences in ontogeny among populations of the same species. These differences appear to reflect contrasting predation pressures among nesting beaches (Gyuris, 1994; Whelan & Wyneken, 2007; Duran & Dunbar, 2015). Lastly, I evaluated differences in

aerobic scope, and identified that larger aerobic scopes appear to indicate that those hatchlings are more active and exert more energy during swimming.

7.1.5 The ontogeny of sea turtle hatchling swimming performance (Chapter 6)

Like metabolic rates, understanding how swimming performance changes over time provides greater insight into the long-term effects of altered incubation conditions. In chapter 6, I measured the change in green sea turtle hatchling swimming behaviour and performance from the frenzy to 24 weeks post-frenzy. Swimming performance, as indicated by mean swim thrust, increased as hatchlings became older. This increase in mean swim thrust was largely driven by an increase in mean maximum thrust (i.e. an increase in the thrust produced per stroke) which was, in turn, largely driven by an increase in hatchling size over time. There was no consistent pattern in the variation in the other swimming attributes from the frenzy to 24 weeks of age. Hatchlings that grow faster are likely to be able to exert increased swimming performance compared to slower growing hatchlings and may experience increased survival rates.

7.2 GENERAL TRENDS

In the following sections, I integrate the results from each experimental chapter presented in this thesis on how moisture concentration during incubation influences hatchling dispersal ability and embryonic development.

7.2.1 Incubation duration and hatching success

The major overall trend that I observed throughout my thesis was that the influence of moisture during incubation on developmental success and hatchling traits was inconsistent. Thus, I suspect that moisture's role in influencing development and hatchling traits is indirect via its influence on other environmental factors. For example, moisture concentration during incubation did not influence incubation duration in flatback, olive ridley or green hatchlings incubated in incubators. However, green hatchlings from natural nests that were maintained at high moisture levels had longer incubation durations than hatchlings from dry nests. Thus, under laboratory conditions where both temperature and moisture were controlled, we observed no variation in incubation duration under different moisture levels. The response of incubation duration is inconsistent among reptiles, with some lizards and snakes, particularly those from arid regions, unresponsive to moisture as an incubation variable (Flatt *et al.*, 2001;

Ji & Du, 2001; Warner & Andrews, 2002; Marco *et al.*, 2004; Du & Shine, 2008). In contrast, in natural nests I controlled moisture levels, yet allowed temperature to fluctuate naturally. Watering directly cools nests and wet nests, experience greater evaporative rates, resulting in cooler incubation temperatures and longer incubation durations (Lolavar & Wyneken, 2017).

In instances where moisture may directly influence hatchling traits and development, the effect of moisture concentrations remained inconsistent. For example, regardless of whether they were incubated in the lab or *in situ*, green and olive ridley hatching success was unaffected by moisture concentrations, while flatback eggs incubated at 4% moisture had lower hatching success than eggs incubated at either 6% or 8%. It is possible that dry incubation conditions resulted in dehydrated flatback eggs and that embryonic development within dehydrated eggs was disrupted (chapter 2). However, flatback eggs are larger than either green or olive ridley eggs and thus, should be most resistant to dry incubation conditions because they contain enough water to successfully develop even in dry conditions (Hewavisenthi & Parmenter, 2000; Hewavisenthi *et al.*, 2001). It is unlikely that eggshell structure determined each species' responsiveness to moisture because eggshell structure and thickness is similar among species (Phillott & Parmenter, 2006). Alternatively, I observed that hatchlings from dry nests took longer to start feeding than hatchlings from wet nests (pers. obs.), suggesting that hatchlings from dry nests have larger yolk reserves. If dry incubation conditions disrupt the conversion of yolk mass into hatchling mass as suggested by Gutzke *et al.* (1987) and Hewavisenthi *et al.* (2001), then this would result in hatchlings with larger yolk reserves or in extreme cases, embryonic mortality. However, I cannot be certain that moisture alone was responsible for these results in flatbacks. The low hatching success I observed was largely driven by two clutches that also had reduced hatching success at other moisture concentrations, indicating that unknown factors may also have been at play.

In conclusion, the effect of moisture on incubation duration appears greatest in natural nests where moisture influences development indirectly, most likely via temperature (Lolavar & Wyneken, 2015) or oxygen concentration (Foley *et al.*, 2006; Cedillo-Leal *et al.*, 2017). Hatching success was generally high over the range of moisture concentrations that I selected, although more extreme concentrations may have a stronger effect on hatching success in both laboratory and natural settings (Mazzotti *et al.*, 1988; Hokit & Branch, 2004; Caut *et al.*, 2010).

7.2.2 Morphology

Like incubation duration and hatching success, the response of hatchling morphology to moisture concentrations during incubation was inconsistent. This suggests that moisture either has minimal effect on hatchling morphology, either directly or indirectly, or the effect of moisture was statistically rather than biologically significant. Moisture concentration influenced flatback head width and olive ridley flipper length at hatching, but did not influence any other measurement. However, at 4 weeks of age, moisture concentration influenced a range of hatchling measurements, although the direction and size of this effect differed among species. The larger effect of moisture at 4 weeks of age, rather than at hatching, suggests that moisture influences growth rates post-hatching, but larger and heavier hatchlings were produced in both dry and wet conditions depending on species. Thus, other factors are likely to have determined hatchling growth rates post-hatching, such as incubation temperatures, food availability and genetics (Dunham, 1978; Niewiarowski & Roosenburg, 1993; Nelson *et al.*, 2004). One consistent result was that green sea turtle hatchlings were not responsive to alterations in moisture concentration during incubation, at least within the range tested here. Green hatchling morphology and locomotor performance were not altered by moisture variation and thus, green hatchlings were the least responsive species to altered nest moisture.

Overall, my results reflect previous studies in other reptile species (chapter 2). Body size is generally optimised at intermediate moisture values and decreases as moisture levels become more extreme (McGehee, 1990; Xiao-long *et al.*, 2012; Brown & Shine, 2018). The inconsistent responses of hatchling morphology to moisture concentrations during incubation may be the result of my 4% and 8% moisture treatments both being on the edge of what developing embryos can reliably tolerate, rather than being over or under that limit. Dry conditions may disrupt the conversion of yolk to hatchling mass (Hewavisenthi *et al.*, 2001), while wet conditions may limit oxygen availability to developing embryos, resulting in reduced hatchling size (Liang *et al.*, 2015; Parker & Dimkovikj, 2019).

7.2.3 Dispersal ability

I measured three indicators of hatchling dispersal ability in this thesis- locomotor performance, thermal tolerance and metabolic rates. The influence of moisture on dispersal ability, like its influence on developmental success and morphology, was largely inconsistent among indicators, and was most likely indirect. However, its effect was not insignificant.

Hatchlings incubated in dry conditions were slower crawlers than hatchlings from wet nests, but swimming performance was unaffected by moisture concentration in any species (chapter 3). The slower crawling speeds of hatchlings from dry nests may be the result of dry incubated hatchlings being more dehydrated than hatchlings from wet nests (Finkler, 1999), although hydration did not influence crawling speeds in the lizard, *Sceloporus undulatus* (Crowley, 1985). Thus, once hatchlings enter the ocean and rehydrate, differences in locomotor performance disappeared. However, I did not detect differences in hatchling hydration among moisture treatment groups in chapter 4, although green hatchling locomotor performance did not respond to moisture concentrations either. Alternatively, hatchlings from dry nests may have greater yolk reserves than hatchlings from wet nests (Hewavisenthi *et al.*, 2001). Hatchlings with greater yolk reserves may have to exert more energy to reach the same crawling speeds as hatchlings from wet nests because they need to carry more 'dead' mass (Miller *et al.*, 1987). Thus, hatchlings from dry nests were slower crawlers. Once hatchlings enter the ocean, the increased buoyancy of the hatchlings makes differences in yolk mass less relevant for swimming speed. My results are similar to those observed in freshwater turtles (Miller *et al.*, 1987; Finkler, 1999), and the tropical keelback snake (*Tropidonophis mairii*) (Brown & Shine, 2006), where higher moisture concentrations resulted in hatchlings that were both faster crawlers and swimmers. In comparison to these tropical reptiles, species from arid zones generally do not respond to moisture concentrations (Flatt *et al.*, 2001; Warner & Andrews, 2002; Du & Shine, 2008). Egg size may influence the sensitivity of species to incubation moisture (Ackerman *et al.*, 1997) , as supported by the responsiveness of olive ridley phenotypes to incubation moisture in chapter 3. However, flatbacks lay the largest eggs out of the species that I tested in chapter 3 and they were more responsive to incubation moisture than greens that lay intermediate sized eggs.

Lastly, moisture is also likely to be influencing hatchling thermal tolerance indirectly, but the effect is stronger in natural nests where temperature is not controlled. Hatchlings that incubated in dry and therefore, warmer nests were able to tolerate warmer temperatures than hatchlings from wet nests. It is possible that hatchlings in warmer nests acclimated to those warmer temperatures and were thus, able to tolerate warmer temperatures than hatchlings from wet nests (Yang *et al.*, 2008). Olive ridley hatchlings in my study had lower thermal tolerance (40.19°C) than those from Drake and Spotila (2002) (41.3°C), potentially because of differences in incubation conditions. Olive ridley hatchlings have similar thermal tolerance

compared to other reptile species, such as the desert box turtle, *Terrapene ornate luteola,* (~41°C) (Plummer *et al.*, 2003), the rock-dwelling velvet gecko, *Amalosia lesueurii,* (38.7-40.2°C) (Dayananda *et al.*, 2017) and chinese softshell turtle, *Pelodiscus sinensis,* (40.9°C) (Sun *et al.*, 2002). Thus, reptile species appear to have conserved thermal tolerance despite substantial variation in geographic range, habitat and life history.

In conclusion, moisture's effect on hatchling dispersal ability varied among indicators. However, moisture appears to be influencing hatchling dispersal in two ways. First, moisture concentrations may influence incubation temperature either directly (Lolavar & Wyneken, 2015) or indirectly via evaporative cooling (Lolavar & Wyneken, 2017). Hatchling traits then respond to altered incubation temperatures resulting in altered hatchling dispersal ability. Second, moisture may be influencing the conversion of yolk mass into hatchling mass. While the mechanism behind this effect is uncertain, hatchlings with greater yolk reserves must carry extra mass that is not contributing to thrust production during terrestrial locomotion. Like developmental success and morphology, it is likely that embryos are relatively resilient to changes in moisture within a certain range, but as conditions become more extreme, moisture will have a stronger effect on hatchling traits.

7.3 ECOLOGICAL IMPLICATIONS

While the response of hatchling traits and developmental success to moisture during incubation varied among species, behavioural stages and indicators of dispersal ability, the effect of moisture has important ecological ramifications for both dispersing hatchlings and adult populations. Here, I detail some potential consequences of altered moisture levels on nesting beaches.

Altered moisture concentrations on nesting beaches are likely to impact certain species more than others. In chapter 3, green sea turtles were the least responsive species to moisture concentrations during incubation. Both flatback and olive ridley hatchlings responded to moisture inconsistently, but generally, olive ridley hatchlings were most sensitive. Thus, olive ridley populations may be at greatest risk of altered moisture regimes on nesting beaches while green sea turtles are likely to be most resilient.

Evaluating the responses of hatchlings among species, drier incubation conditions are likely to result in hatchlings that are slower crawlers (chapter 3) and therefore, are at greater risk of predation during dispersal from the nest to the ocean (Husak, 2006b; Husak, 2006a). However, these hatchlings are also likely to have greater thermal tolerance than hatchlings from wet nests (chapter 4). The ecological significance of these responses will depend on the characteristics of each nesting beach. Hatchlings dispersing on predator-dense beaches are likely to benefit more from faster crawling speeds to escape predators. Conversely, hatchlings on black sand beaches that are warmer than white sand beaches (Hays *et al.*, 2001) may benefit more from increased thermal tolerance. Despite the importance of hatchling crawling speed and thermal tolerance, hatchling survival is largely dictated in the ocean where predation rates are generally higher than on land (Gyuris, 1994; Santidrián Tomillo *et al.*, 2010). During the frenzy or the first 24 hours of dispersal, moisture is unlikely to have a large influence on hatchling survival because frenzy and post-frenzy swimming performance was not altered by moisture concentrations during incubation (chapter 3).

In conclusion, drier incubation conditions may be beneficial for hatchlings once they reach the ocean because they have greater thermal tolerance. However, dry incubation conditions may also make hatchlings more susceptible to predation during terrestrial dispersal on nesting beaches. Additionally, as natural nests become drier, they are also likely to become warmer (Lolavar & Wyneken, 2015), potentially resulting in smaller hatchlings that are weaker crawlers and swimmers (chapter 2). Overall, hatchlings appear to be relatively resilient to variation in moisture levels within certain ranges, but as conditions become more extreme, we may observe stronger effects on hatchling traits.

The effect of moisture on hatchlings during incubation will also have important consequences for adult sea turtles. First, as described above, altered hatchling traits and developmental success will influence hatchling survival and recruitment into adult populations, affecting population dynamics and viability. Second, the optimal time for females to nest may be altered by variation in moisture concentrations on nesting beaches. Below, I discuss how both hatchling recruitment and adult nesting behaviour may be altered by variation in nest moisture concentrations.

The influence of moisture on hatchling recruitment is likely to be limited overall because moisture concentrations during incubation did not alter hatchling swimming performance, which is where the majority of predatory events occur for dispersing sea turtles (Gyuris, 1994; Santidrián Tomillo *et al.*, 2010). However, this is not to say that it will have no effect. The effect of altered moisture concentrations on hatchling recruitment will vary depending on a number of factors, such as species' behaviours and nesting beach characteristics. Species and populations that experience a decrease in hatchling survival and recruitment are likely to also experience reduced population viability (Schwanz *et al.*, 2010). Although I did not detect an effect of moisture during incubation (chapter 3), moisture has been shown to have an influence on hatchling phenotypes and growth in other studies (Robbins & Warner, 2010). However, the short and long-term effects of moisture are inconsistent (Alberts *et al.,* 1997; Erb *et al.,* 2018) and generally, the effects of incubation temperatures are longer than lasting than those of moisture (Elphick & Shine, 1998; Booth, 2006; Du *et al.,* 2007).

The response of nesting females to altered moisture conditions will depend on how much of an influence moisture has on hatchlings traits, the range of moisture concentrations available to nesting females and species differences. In species, such as green sea turtles, that are more resilient to variation in moisture, females are less likely to respond to changes in moisture regimes on nesting beaches compared to species that are more sensitive. However, in species that are sensitive to moisture concentrations during incubation, nesting females may be able to maximise their reproductive fitness by altering their nest site selection and nesting phenology to optimise the incubation conditions experienced by their offspring. Sand moisture content has been shown to vary both spatially and temporally (chapter 2). Spatially, females are able to select wetter nest sites by depositing eggs closer to the ocean, away from vegetation or in deeper nests (Ackerman *et al.*, 1997; Wood *et al.*, 2000; Conrad *et al.*, 2011). Temporally, females can lay during the wet season or may time their nesting to coincide with rainfall events, as seen with females adjusting the timing of nesting with sea surface temperatures (Dalleau *et al.*, 2012; Lamont & Fujisaki, 2014). However, the ability of nesting females to select various incubation conditions for their nests will depend on the range of incubation conditions available on nesting beaches. Beaches that are homogenous limit the ability of females to select optimal incubation conditions (Kamel & Mrosovsky, 2006; Mcnew *et al.*, 2013)

In conclusion, adult populations are likely to be influenced by altered moisture concentrations in two ways. First, hatchling traits will respond to altered moisture concentrations, potentially influencing hatchling recruitment and population dynamics. Secondly, females may alter their nesting behaviour in order to optimise the incubation conditions of their offspring. However, the response of adult populations will depend on each species' traits and the specific characteristics of nesting beaches. Lastly, the response of both hatchlings and adults to variation in moisture concentration may be limited within current moisture ranges, although as moisture concentrations become more extreme under climate change, population dynamics and hatchling recruitment may respond more strongly.

7.4 LIMITATIONS AND FUTURE DIRECTIONS

In a single thesis, it is impossible to address every idea or question due to a lack of time and resources. Furthermore, the questions I was able to address have led to new avenues of research that are worth pursuing. Here, I discuss potential future research directions as well as limitations to the studies that I was able to undertake.

In chapter 2, I reviewed the response of hatchling traits and developmental success to a variety of environmental factors. However, my review only considered the responses of hatchlings in oviparous reptiles. The responses of other oviparous species or viviparous species may differ from those of reptiles. Furthermore, future studies should consider investigating environmental factors other than temperature, such as moisture, oxygen and environmental contaminants, like salinity. Lastly, research on environmental factors generally focused on certain species in the testudines and squamates, but largely ignored other species and taxa. Future studies should expand their focus to more species.

This thesis is a preliminary investigation into the effects of moisture on hatchling dispersal ability. Thus, I incubated eggs at various moistures and maintained constant temperatures. While this isolated the effects of moisture, it did not provide a complete understanding of how incubation conditions in natural nests impact hatchling traits. Additionally, I incubated eggs separately, in a single layer within incubators rather than in a clutch with depth, like normal nests. This was to minimise the risk of bacteria and fungi spreading from dead eggs to live ones, and also to ensure maximum control over the moisture surrounding the eggs. Eggs within a natural nest can experience considerable differences in temperature and humidity

based on their position in the nest. By incubating eggs completely surrounded by sand rather than in a clutch of eggs, my incubation set-up did not fully replicate natural nests, and may have had unknown effects on development. In contrast, when incubating eggs in a natural setting, it was impossible for this study to regulate both the temperature and moisture concentration during incubation. Regardless of whether future studies incubate eggs in incubators or natural nests, the next step for this research is to measure the response of hatchling dispersal ability to interacting incubation conditions, particularly at extreme temperatures and moisture concentrations. This would provide further insight into how hatchling dispersal ability and thermal tolerance may respond under climate change, and under which conditions dispersal ability and thermal tolerance peak or begin to be negatively impacted by further increases or decreases in temperature and/or moisture.

For all three species that I incubated, tested and housed at Monash University in this thesis, I transported the eggs using hypoxia, when I sealed the eggs in vacuum-seal bags to arrest embryonic development. While all eggs were exposed to the same transport method, some eggs were sealed for up to 3 days while others were only sealed for 24 hours. When comparing hatchling traits based on how long eggs were maintained in hypoxia, there was no difference in any trait that I measured. However, it remains possible that hypoxic transport of the eggs influenced hatchling development and altered hatchling traits compared to hatchlings from natural nests. Flatback hatchlings maintained in hypoxia for 5 days were larger and faster than hatchlings incubated completely in normoxia (Rings *et al.*, 2014), thus hatchlings in this study may also be larger or faster than they would have been in natural nests.

I hypothesised that hatchling hydration may play a role in determining differences in crawling speeds, self-righting ability and thermal tolerance among moisture concentrations during incubation. Once hatchlings enter the ocean, they drink large quantities of water and rehydrate. Thus, differences among moisture treatments disappear. However, I did not measure hatchling hydration when testing locomotor performance and cannot be certain that hatchling hydration is the mechanism influencing hatchling locomotor performance. Future investigations into the effect of moisture on locomotor performance should consider measuring hatchling hydration during emergence and dispersal to fully understand the role that hydration plays. When I did measure hatchling hydration in chapter 4, I did not measure blood osmolarity, which may have provided further insight into the role that moisture during

incubation plays in determining hatchling hydration and the consequent effect that hydration has on thermal tolerance. Additionally, packed cell volume and total protein are both indicators of the relative hydration of hatchlings, but there can be variation in both packed cell volume and total protein measurements among even fully hydrated individuals (Bolten & Bjorndal, 1992; Wicks & Schultz, 2008; Kimble & Williams, 2012). Generally, studies take baseline measurements to use as an indicator of hatchling hydration (Boyd, 1981; Bak *et al.*, 2017), but this was not possible with developing embryos. An alternative solution may have been to hold hatchlings post-testing, allow them to rehydrate and then create a baseline measure of hydration post-hatching, although this was not possible in this study.

While I found that hatchling hydration did not have a strong influence on thermal tolerance, my results did suggest that incubation temperature was responsible for variation in thermal tolerance, but the mechanisms that drove this response remain unclear. Is temperature influencing the development of hatchlings resulting in long-term thermal adaptation or are embryos acclimating to increased incubation temperatures as seen in other free ranging organisms? Does temperature influence thermal tolerance throughout incubation or are the temperatures in the last few days pre-emergence the main driver of thermal tolerance? Would a decrease in temperature during emergence override the thermal tolerance of hatchlings that were incubated in warm conditions throughout the majority of incubation? These questions will need to be investigated further to fully understand the role that temperature plays in determining thermal tolerance in sea turtle hatchlings. Future studies will need to manipulate incubation temperatures in order to investigate how and when changes in temperature influence thermal tolerance and what the limits of this relationship are.

When measuring hatchling swimming performance, I placed hatchlings in vests attached to a load cell with monofilament line. As the hatchlings swam, the load cell recorded their thrust production per stroke. While I made sure that the vest did not impede flipper movement, it is impossible to say that the vest did not influence hatchling swimming behaviours. Also, sea turtle hatchlings utilise numerous cues including light, geomagnetic fields and waves to orient themselves during dispersal (Lohmann *et al.*, 1990; Lohmann, 1991; Tuxbury & Salmon, 2005). These cues may not be present or may be altered in laboratory settings, resulting in hatchlings spending more time orienting themselves and less time swimming (Salmon & Wyneken, 1987). Thus, my measurements may not accurately reflect natural swimming behaviours, such as the proportion of time that hatchlings spend power-stroking.

6330 However, the methodology employed in this study is currently the most useful technique for
6331 measuring swimming performance and isolating individual swimming behaviours.
6332
6333 Measuring thrust production is not the same as measuring swimming speed. Swimming speed
6334 is determined by the amount of thrust being produced and the amount of drag produced by
6335 each hatchling (Prange, 1976). Drag is largely determined by the size and shape of an object
6336 as it passes through a medium, in this case water. When hatchlings are a similar size and
6337 shape, drag is likely to be similar (Watson & Granger, 1998; Jones *et al.*, 2011; Jones *et al.*,
6338 2013) and therefore, as seen in chapter 3, thrust production is a strong indicator of relative
6339 swimming speed. However, in this chapter, I compared the thrust production of green
6340 hatchlings at multiple ages where their size varied considerably. Thus, the amount of drag
6341 being produced by hatchlings is likely to have differed among age groups. Considering that
6342 hatchlings remained a similar shape as they grew, it is unlikely that drag production changed
6343 drastically. So, while swim speed may not have increased at the same rate as thrust
6344 production, because drag also increased as hatchlings grew larger (Jones *et al.*, 2011), thrust
6345 production remained a strong indicator of swimming performance.
6346
6347 Chapter 6 measured the ontogeny of green sea turtle hatchling swimming performance from
6348 hatching to 24 weeks of age. When measuring hatchling swimming performance, I recorded
6349 thrust production and other behaviours in short 2 hour (frenzied hatchlings) or 30-minute
6350 bursts (4, 12 and 24-week-old hatchlings). Thus, changes in swimming performance reflected
6351 the ability of hatchlings to swim in specific bursts. In natural conditions, green hatchlings
6352 may only swim in 5-minute bursts or may swim continuously during daylight hours (Salmon
6353 & Wyneken, 1987; Salmon *et al.*, 2009). Therefore, my measurements of swimming
6354 performance may not be ecologically relevant. Although, predation rates are generally
6355 highest within the first few hours of the frenzy (Gyuris, 1994) and activity levels generally
6356 decrease post-frenzy (Salmon & Wyneken, 1987), indicating that my chosen trial lengths are
6357 ecologically relevant. The lack of cues for orientation in my laboratory may also be partly
6358 responsible for hatchlings maintaining high levels of swimming activity post-frenzy (Salmon
6359 & Wyneken, 1987). My results do provide a measure of the physiological capacity of
6360 hatchlings to exert continuous swimming efforts even if they may not normally do so under
6361 natural conditions. Measuring swimming performance for 24 hours at each age would
6362 provide us with a greater understanding of how hatchlings spend their time and provide more

realistic insights into their natural behaviours. I was only able to house green hatchlings for 24 weeks and could not measure the ontogeny of swimming performance in olive ridley or flatback hatchlings beyond 4 weeks of age. Thus, I could not compare long-term changes in swimming performance among species. Expanding upon the number of species tested would provide a greater understanding of differences in life history among species.

Developing methodologies and technologies to measure swimming performance and metabolic rates *in situ* will aid in fully understanding how incubation conditions influence hatchling survival and dispersal. Hatchling survival and dispersal success is determined by a number of interacting factors including swim performance, thermal tolerance and metabolic rates. Expanding future research to investigate not only the effects of interacting incubation conditions, but also interacting hatchling phenotypes, will more accurately reflect natural conditions. Ideally, I would have measured each species' metabolic rates, locomotor performance and thermal tolerance simultaneously. By measuring oxygen consumption and swimming performance simultaneously, Booth (2009) was able to directly correlate metabolic rate with thrust production, as well as compare the performance of each individual hatchling. By measuring both oxygen consumption and swimming performance in progressively warmer water temperatures, I would be able to simultaneously measure the interaction between thermal tolerance, locomotor performance and oxygen consumption. I would also measure these interactions at multiple ages and in multiple species. Thus, I would be able to compare the ontogeny of swimming performance and metabolic rates in multiple species, investigate how thermal tolerance impacts hatchling dispersal ability, as well as evaluate the effect of incubation conditions on hatchling dispersal ability.

In chapter 5, I compared the frenzy and post-frenzy metabolic rates of five different sea turtle species and found that the ontogeny of metabolic rates largely reflected differences in life history. I was able to compare the metabolic rates of five species because I included previously unpublished metabolic rate data on green, loggerhead and leatherback hatchlings. However, utilising this data meant that the metabolic rates in this study were collected using three different methodologies. While the method used should not alter my measurements of oxygen consumption, it may influence hatchling behaviour slightly and there will inevitably be slight differences among systems. The differences in methodology also resulted in oxygen consumption being measured at different activity levels. Compared to the additional data, I

did not measure metabolic rate during crawling and I measured maximal metabolic rate rather than metabolic rate during routine swimming. Maximal metabolic rate is measured as the hatchlings are swimming with maximum effort while active metabolic rate is measured as hatchlings swim at their own natural pace, without encouragement or prodding. Thus, the two measurements, though similar, are not the same. Differences in methodology meant that post-frenzy hatchlings were tested at slightly different ages. The olive ridley, flatback and green hatchlings that I collected (closed respirometry 2017/18) were tested at 4 weeks of age, but hatchlings tested using closed respirometry (2010) and open flow respirometry (1996 & 2000) ranged from 12 to 45 days. Lastly, I measured metabolic rates at a single water temperature, but ectotherm activity levels and metabolic rates have been shown to vary with temperature (Wang *et al.*, 2002; Clark *et al.*, 2006; Parker & Dimkovikj, 2019). The effects of temperature on metabolic rates may be greater in hatchlings (Booth & Evans, 2011) than in larger juveniles that showed reduced responses to seasonal variation in ocean temperature (Southwood *et al.*, 2003; Southwood *et al.*, 2006). Changes in beach temperatures likely occur simultaneously with changes in sea temperatures, so future studies should consider the effects of not only altered incubation conditions but also dispersal conditions.

I found that differences among populations, such as predation rates (Gyuris, 1994; Duran & Dunbar, 2015), may also contribute to variation in the ontogeny of metabolic rates. While previous studies identified variation among species (Wyneken, 1996; Jones *et al.*, 2007), and related that variation to differences in life history, no studies did so at the population level within species. Thus, future studies should consider not only variation among species, but also differences in predation rates and other selective pressures among populations. Ideally, hatchling metabolic rates would be measured continuously and *in situ* as they disperse. By simultaneously tracking mortality rates and dispersal distance and speeds, I would be able to gain a more complete picture of how metabolic rates are fluctuating during dispersal and the impact this has on hatchling survival. It would also allow me to monitor activity levels and metabolic rates over a longer time period, as hatchlings grow and become juveniles. Measuring the ontogeny of metabolic rates until hatchlings become juveniles would allow me to make stronger inferences on life history variation among species and evaluate how seasonal variation in temperature, day length and food availability influence behaviour and physiology (Southwood *et al.*, 2003; Southwood *et al.*, 2006; Duran & Dunbar, 2015). However, the technology to track hatchlings is limited and current methods for measuring

metabolic rates long-term and *in situ* do not provide the fine scale information required to differentiate among separate activity levels (Jones *et al.*, 2009). Thus, technology needs to improve before such experiments can be considered.

7.5 CONCLUSIONS

Moisture concentration during incubation appears to have an indirect, yet important influence on hatchling dispersal traits in sea turtles. Drier incubation conditions produced hatchlings that were slower crawlers and were slower to self-right. However, moisture did not influence the swimming performance of hatchlings, potentially limiting its overall effect on population dynamics. Furthermore, hatchlings from dry nests had greater thermal tolerance, possibly because they became acclimated to higher temperatures in dry nests. Thus, hatchlings that emerge from nests laid during droughts or during the dry season, may be at greater risk of predation as they crawl to the ocean, but are better equipped to handle high sand temperatures during this period. The impact of moisture during incubation was not consistent among behavioural stages, activity levels or species. More research is required to fully elucidate how alterations to incubation conditions, including moisture, impact hatchling recruitment and population dynamics. Considering the inconsistency of moisture's effect, it is likely that sea turtle hatchlings are relatively resistant to variation in moisture within certain ranges, although as conditions become more extreme, hatchling traits may respond more strongly. Overall, this thesis has contributed new knowledge to our understanding of how incubation conditions influence hatchling dispersal ability and thus, hatchling survival. Additionally, by comparing the ontogeny of dispersal ability in multiple species, this thesis has provided new insight into variation in life histories among species and populations, as well as how incubation conditions may influence the dynamics and viability of sea turtle populations.

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Appendix I

Supplementary material for Chapter 3

Vacuum-sealed o*live ridley eggs arrive at Monash*

Photo taken by Christopher Gatto.

Supplementary Table 3.1: Statistical results from linear mixed effects model on the effect of moisture during incubation on locomotor performance. We also present the amount of variance explained by random effects.

			Moisture content (fixed effect)			***Variance explained by random effects***		
	Species	***Week***	*Df (NumDF, DenDF)*	*F-value*	*p-value*	*Clutch*	*Temperature (crawling trial)*	*Temperature (swimming)*
Time to self-right	Green	0	1,58	2.1	0.151	0%	0%	
	Olive ridley	0	**1,68.7**	**45.6**	**>0.001**	44.34%		
	Flatback	0	**1,74.7**	**16.8**	**>0.001**	8.22%	0%	
Number of successful self-righting attempts	Green	0	1	2.8	0.097			
	Olive ridley	0	**1**	**52.95**	**<0.001**			
	Flatback	0	**1**	**31.6**	**<0.001**			
Mean crawling speed	Green	0	1, 4.6	1.1	0.352	19.82%	2.6%	
	Olive ridley	0	**1, 70.6**	**10.6**	**0.002**	18.34%	0%	
	Flatback	0	**1, 75.2**	**2.6**	**0.11**	1.26%	0%	
Mean swim thrust	Green	0	1,55	0.4	0.522	0.61%		0%
		4	1,54.6	0.6	0.439	12.76%		2.56%
	Olive ridley	0	1,69.2	0.2	0.67	36.53%		0%
		4	1,67.1	0.05	0.826	13.31%		0%
	Flatback	0	1,74.4	0.1	0.714	12.39%		0%
		4	1,77	0.3	0.591	0%		0%

Proportion of time spent power stroking	Green	0	1,56.4	0.7	0.402	0%		13.32%
		4	1,55	0.1	0.748	25.38%		0%
	Olive ridley	0	1,69.8	0.3	0.597	27.07%		0%
		4	1,67.9	0.7	0.409	1.51%		0%
	Flatback	0	1,73.8	0.2	0.63	19.93%		0%
		4	1,76.9	0.1	0.719	0%		2.79%
Powerstroke frequency	Green	0	1,55	2.4	0.124	7.99%		0%
		4	1,54.2	0.4	0.536	8.92%		29.81%
	Olive ridley	0	1,71.5	1.9	0.17	1.42%		0%
		4	1,62.2	0.8	0.377	0%		6.52%
	Flatback	0	1,75.6	1.8	0.183	3.7%		0%
		4	1,73.7	0.9	0.341	5.2%		6.5%
Duration of power stroking bouts	Green	0	1,55.8	0.7	0.41	0%		26.12%
		4	1,55	0.6	0.457	13.34%		0%
	Olive ridley	0	1,71.3	2.4	0.124	3.86%		0%
		4	1,62.2	0.3	0.606	4.17%		0%
	Flatback	0	1,75.3	0.5	0.462	5.74%		0%
		4	1,75	0.1	0.756	0%		0%
Mean maximum thrust	Green	0	1,53.9	0.3	0.598	0.97%		2.81%
		4	1,55	2.4	0.128	6.92%		0%
	Olive ridley	0	1,68.3	1.4	0.24	43.21%		0%
		4	1,62.3	0.2	0.646	1.98%		0%
	Flatback	0	1,76.4	1.9	0.169	0%		10.11%
		4	1,75	1.3	0.255	0%		0%

Supplementary Table 3.2: Statistical results from linear mixed effects model on the change in swimming performance attributes over time. We also present the amount of variance explained by random effects.

	Species	Week (fixed effect)			Variance explained by random effects			
		Df	*F-value*	*p-value*	*Clutch*	*Moisture content*	*Water temperature*	*Hatchling ID*
Mean swim thrust	Green	**1,36.1**	**61.5**	**<0.001**	2.82%	0%	2.4%	0%
	Olive ridley	1,72.5	0.08	0.78	12.85%	0%	0%	1.76%
	Flatback	**1,151.3**	**48.2**	**<0.001**	3.34%	0%	0%	0%
Proportion of time spent power stroking	Green	**1,89.2**	**13.5**	**<0.001**	0%	0%	9.68%	0%
	Olive ridley	1,71.5	1.2	0.29	12.71%	0%	0%	4.33%
	Flatback	**1,151.4**	**49.2**	**<0.001**	3.37%	0%	0%	0%
Powerstroke frequency	Green	**1,86.6**	**74.8**	**<0.001**	2.68%	0%	7.43%	0%
	Olive ridley	1,49.5	0.4	0.55	1.63%	0.02%	2.16%	8.34%
	Flatback	**1,149.4**	**68.6**	**<0.001**	1.46%	4.78%	0%	0%
Duration of power stroking bouts	Green	1,34.6	**2.85**	**0.1**	4.37%	9.12%	1.05%	25.24%
	Olive ridley	1,135.6	2.3	0.13	0%	0.54%	0%	0%
	Flatback	**1,149.7**	**40.2**	**<0.001**	0.90%	0%	0%	0%
Mean maximum thrust	Green	**1,59**	**450**	**<0.001**	0%	0%	0%	17.29%
	Olive ridley	1,131.1	0.4	0.52	12.84%	0%	0%	0%
	Flatback	**1,74.7**	**18**	**<0.001**	0%	0.15%	0.32%	4.88%

Supplementary Table 3.3: Statistical results from linear mixed effects model on the differences in locomotor performance between species. We also present the amount of variance explained by random effects.

		Week (fixed effect)			Variance explained by random effects			
	Week	*Df*	*F-value*	*p-value*	*Clutch*	*Moisture content*	*Temperature (crawling trial)*	*Temperature (swimming trial)*
Time to self-right	0	2, 11.98	1.9	0.194	18.81%	13.92%	0%	
Number of successful self-righting attempts	0	2	1.2	0.312				
Mean crawling speed	0	**2, 13.8**	**63.3**	**<0.001**	10.63%	4.55%	0%	
Mean swim thrust	0	**2, 12.9**	**54.8**	**<0.001**	9.01%	0%		0%
	4	**2, 12.9**	**180.5**	**<0.001**	1.91%	0%		0%
Proportion of time spent power stroking	0	2, 13.1	2.0	0.172	17.58%	0%		1.61%
	4	**2, 2**	**31.5**	**0.031**	0%	1.28%		1.41%
Powerstroke frequency	0	**2, 11.5**	**25.0**	**<0.001**	3.66%	0.67%		0%
	4	**2, 11.7**	**23.8**	**<0.001**	5.97%	0%		0%
Duration of power stroking bouts	0	2, 14.5	0.1	0.916	3.2%	0.03%		12.77%
	4	**2, 3.5**	**37.1**	**0.004**	4.67%	0%		0.02%
Mean maximum thrust	0	**2, 9.7**	**252.6**	**<0.001**	5%	0%		0%
	4	**2, 12.4**	**350.5**	**<0.001**	1.96%	0.96%		0%

Appendix II

Supplementary material for Chapter 5

Basking green sea turtles on the North shore in Hawaii.

Photo taken by Christopher Gatto

Supplementary methods

Closed respirometry: flatback, green and olive ridley sea turtle hatchlings

We collected olive ridley (*Lepidochelys olivacea*) and flatback sea turtle (*Natator depressus)* eggs in Australia from the Tiwi Islands, NT and Curtis Island, QLD in 2017 and 2018, respectively. We patrolled nesting beaches at night looking for nesting females and collected the eggs as they were laid or just after oviposition if we found the female covering the nest. We collected 30 eggs from each of 6 females per species. The eggs were vacuum-sealed in bags following the protocol of Williamson *et al.* (2017) to maintain embryonic arrest. Eggs were vacuum-sealed within 1 hour of oviposition and were sealed for a total duration of 24-72 hours. The sealed bags were placed in a cooler lined with vermiculite or bubble wrap and containing ice packs. We then transported the eggs to Monash University, Melbourne, VIC where they were placed into incubators (1602-N Hovabator).

Eggs were ¾ buried in sand and incubated at each species' pivotal temperature and at moisture concentrations that ranged from 4% to 8% moisture w/w. Incubator temperature was monitored daily using fast response temperature probes (PASCO PS-2135) buried next to the eggs and we maintained moisture gravimetrically by drying samples of sand and adding evaporated water with a spray bottle. We removed eggs that turned yellow or showed signs of fungus or mould to avoid contamination of other eggs. Once all eggs had formed white spots, we fully covered the eggs with sand.

Green sea turtle eggs *(Chelonia mydas)* were collected from Kijal beach, Malaysia, 42km from the Lang Tengah Turtle Watch hatchery in 2018. The eggs were transported to the shaded hatchery in buckets lined with sand and buried in the centre of a $1m_2$ plot with the bottom of the nest at a depth of 70cm. We collected entire clutches from 20 nesting females and all nests were reburied within 6 hours of oviposition. We measured moisture with a probe (PASCO ECH_2O EC-5) and each clutch was maintained between 4% and 8% moisture (v/v) by adding water with a watering can at the surface. The amount of water required each day was determined during a pilot study in which we watered empty plots with various volumes of water and monitored changes in sand moisture concentration.

After emerging from the eggs, olive ridley and flatback hatchlings were given 48 hours to internalise their yolk sac. We then removed them from their incubators for testing. Green sea turtle hatchlings were collected for testing as they emerged from the surface of the hatchery nes. We marked hatchlings on the carapace with unique patterns using non-toxic nail polish and measured hatchling mass using electronic scales (±0.001g).

We measured both resting (RMR) and maximal metabolic rate (MMR) of hatchlings. First, we tested RMR by placing hatchlings in a small closed chamber (~375mL) with an O_2 probe (PASCO PS-6524) recording the change in O_2 concentration. We used soda lime (Scharlau, Australia) and Drierite™ (Hach, Australia) to remove CO_2 and H_2O from the air, respectively. We calibrated the O_2 probe to the ambient O_2 concentration (20.9%) before each trial began and we checked the system for leaks using N_2 gas. We began trials once the hatchling became still (generally within 5 min) and restarted trials if the hatchling became active or agitated. Hatchlings remained in the respirometry chamber for 20 min. Olive ridley and flatback hatchlings were tested in a controlled temperature room set to 25°C and green hatchling testing occurred in the Lang Tengah Turtle Watch headquarters at ambient temperature (27.5 ± 1.2°C). Oxygen consumption was calculated by subtracting the O_2 concentration at the end of each trial from the concentration at the start of each trial.
Next, we tested hatchling MMR when hatchlings swam maximally. We placed a glass chamber upside-down in seawater, creating a pocket of air between the water and the chamber (~1000mL). We pumped air from the chamber at ~200 ml min^{-1} over an O_2 probe (PASCO PS-2126A) sampling at 2Hz before returning the air to the chamber. The air was scrubbed using soda lime to remove CO_2 and drierite to remove H_2O before passing over the O_2 probe. Hatchlings were placed in elasticised harnesses and tethered to the top of the chamber with fishing lines so they could swim but not touch the sides of the chamber. We placed a light at one end of the chamber to encourage the hatchling to swim unidirectionally. Trials lasted 15 min and to ensure the hatchlings swam maximally, we tapped them on the back of the carapace using a bent piece of wire passed underneath the chamber, encouraging a flight response (Jones *et al.*, 2007). Water temperatures for maximal metabolic rates were 26.3 ± 0.4°C for flatback and olive ridley hatchlings, and 26.6 ± 1°C for green hatchlings.

Olive ridley hatchlings were tested during the frenzy (0 weeks of age, sample size (N)=74, mass ± se 16.46 ± 0.21g) and post-frenzy (4 weeks of age, N=70, 19.39 ± 0.28g), green hatchlings were tested during the frenzy only (N=95, 21.37 ± 0.21g) and flatback hatchlings were tested during the frenzy (N=80, 40.39 ± 0.31g) and post-frenzy (N=79, 63.32 ± 0.52g). Olive ridley and flatback hatchlings were housed in 3L and 10L plastic tanks or in glass tanks separated with egg crating (12.5mm grid, Aquasonic, Australia). Tanks were kept clean by a continuous flow-through system consisting of a drum filter (Faivre 60 series, Faivre, France), fluid sand bed filters (RK2 systems, USA), a protein skimmer (RK10AC, RK2 systems, USA), a UV filter (240W UV steriliser, Emperor Aquatics, USA) and an ozone steriliser (RK300MG, RK2 systems, USA). Water quality was monitored daily using OxyGuard hand-held monitors (Technolab, Australia). Water temperature was maintained between 26 and 27°C using a heater (3kW heater, Shego, Germany) and a chiller (FBT175SSD, Toyesi, Australia). Animals were maintained under a day/night cycle of 12 hours and provided with UV lighting (Exo Terra Repti Glo 5.0 25W). Turtles were fed with commercial turtle pellets (4mm Marine float range, Ridley Aquafeed).

After testing was completed, 4-week-old olive ridley and flatback hatchlings were transported back to the site of collection and released. Green hatchlings were released on the beach adjacent to the Lang Tengah Turtle Watch hatchery within 24 hours of emerging. Eggs were collected under Queensland scientific purposes permit WITK18685417 (flatbacks), Northern Territory permit to take wildlife 62703 (olive ridleys) and Terengganu State Fisheries Office approval to carry out research work SEATRU/RES/17/01 (greens). Experimental procedures were conducted under approval SEATRU/RES/17/01 for green sea turtles and under Victorian research permit 10008208 for flatback and olive ridley hatchlings. All procedures were approved by the Monash University School of Biological Sciences Animal Ethics Committee (approval BSCI/2018/08 for green sea turtles and BSCI/2016/23 for olive ridley and flatback sea turtles). Egg collection and hatchling release of olive ridley hatchlings was conducted with the permission and assistance of the Tiwi Land Council and the Science Reference Council.

Closed respirometry- leatherback, loggerhead and green sea turtles

Hatchlings were collected from natural nests laid in Boca Raton, Florida, USA throughout June, July and August of 2010. Hatchlings were housed at Florida Atlantic University in

clutch-specific tanks with separate water and filter systems for each clutch. Leatherbacks *(Dermochelys coriacea)* were housed using a tether system that prevented hatchlings from touching the side of the tanks while still allowing swimming in any direction, following the protocol of Jones *et al.* (2000). Hatchlings in their frenzy were naïve to the water prior to the study. They were held in Styrofoam™ boxes with nest sand and placed in a quiet dark room prior to testing. For post frenzy tests, green and loggerhead (*Caretta caretta)* hatchlings were individually housed in plastic baskets suspended within the larger holding tank. The baskets allowed seawater to circulate via small holes in the side of the baskets but kept hatchlings physically separated. Tank water was approximately the same temperature as the ocean water and all tests were conducted at 24°C-28°C. Hatchlings were fed daily after day 3 (loggerheads) and day 5 (leatherbacks) and were provided with 12 hours of full-spectrum radiation daily by UV lighting. Hatchlings were released offshore following testing.

Testing occurred in a 35cm × 35cm Plexiglass™ respirometry chamber or a glass and acrylic chamber (loggerheads and leatherbacks) that was 50.8cm × 25.4cm. Chambers were filled with seawater so that an air space of 1-2cm in height was left between the chamber lid and the water. Thus, the air volume during testing could be calculated from the chamber cross-sectional area and the height of the air space. We replaced the seawater with fresh, autoclaved seawater allowed to come to room temperature between clutches.

Hatchlings were randomly selected from each clutch for testing. Leatherback hatchlings were tested at 20 days (sample size (N)=4, mass ± se 68.02 ± 5.47g), 23 days (N=6, 61.56 ± 3.32g) and 44 days (N=1, 99.21g). Loggerhead hatchlings were tested at 6 days (N=5, 16.81 ± 0.23g), 43 days (N=2, 60.68 ± 7.95g), 51 days (N=2, both 89.87) and 52 days (N=1, 53.65g). Green turtle hatchlings were all tested on the day of emergence (N=6, 24.6 ± 0.18g). Tank temperature was recorded before each trial (range: 24-30°C). Each hatchling was fitted with a Velcro™ strip attached with Vetbond (3M, USA), slightly caudal to the longitudinal midpoint of the carapace. We attached one end of a monofilament line to the Velcro strip and the other to the top of the respirometry chamber. Thus, hatchlings could swim in any direction without touching the walls of the chamber. Hatchlings were allowed to acclimate for 30 min, while the respirometry system was bypassed and sampled ambient air. Once the hatchling had acclimated, the system was reconnected and air of known O_2 and N_2 partial

pressure flowed through a Mass Flow Controller (Sierra Side-Trak 840). Air was scrubbed of water vapor (Drierite water absorbent, W.A. Hammond DRIERITE, Xenia, OH, USA) before being drawn through an Applied Electrochemistry O_2 Analyser S-3A (AEI Technologies, Pittsburgh, PN, USA). Data from the mass flow controller and oxygen analyser were recorded at the start and the end of the trial and was analysed using DataCan V Data Acquisition and Analysis Software and Hardware (Sable Systems International, Las Vegas, NV, USA). Air was then re-circulated back through the closed system configuration. Respirometer calibration was done using the N_2 dilution technique (Fedak et al., 1981). VO_2 data were corrected for analyzer drift and to STP. Tank temperature was recorded before each trial (range: 24-30°C). Leatherback hatchling testing lasted for an average of 55 min, green hatchlings for 20 min and loggerheads for an average of 27 min.

Hatchling collection, testing and housing were conducted under FAU IACUC protocol A10-18 and Florida Sea Turtle Permit #073.

Open flow respirometry- leatherback, loggerhead and green sea turtles

Green, loggerhead and leatherback turtle hatchlings were collected from natural nests laid in Boca Raton, Florida, USA throughout June, July and August of 1996 and 2000. Additional leatherback hatchlings were collected from natural nests laid in Boca Raton, Hillsboro beach, Juno Beach and Jupiter Beach, Florida, USA. Hatchlings were housed at Florida Atlantic University in clutch-specific tanks with separate water and filter systems for each clutch. Green and loggerhead hatchlings were kept in individual baskets within the larger holding tank. The baskets allowed seawater to circulate via small holes in the side of the baskets but kept hatchlings physically separated. Leatherbacks were housed following the protocol of Jones *et al.* (2000) as described above. Tank water was approximately the same temperature as the ocean water and all tests were conducted at 24°C-28°C. Hatchlings were fed daily after day 3 (loggerheads) and day 5 (leatherbacks) and were provided with 12 hours of full-spectrum radiation daily by UV lighting. Hatchlings were released offshore following testing.

When measuring resting metabolic rates, hatchlings were placed in an approximately 470mL black container (~10 cm × 7.5 cm, approximately 470mL) closed with a large rubber stopper fitted with air intake and outflow. Each turtle was allowed to acclimate for 30 min, and hatchling movement was minimised in the small container. Once hatchlings were inactive

(based on no audible sound from the claws or flippers on the glass), we closed the container, and began measuring the O_2 consumption and measured for 90 min. If hatchlings became active, we restarted metabolic measurements.

For measurements of metabolic rates during crawling (CMR) and routine swimming metabolic rate (AMR), testing occurred in the same 26 L tank fitted with an acrylic respirometry chamber and sealed with petroleum jelly. During CMR testing, hatchlings were allowed to crawl on a textured glass floor. During testing of routine swimming metabolic rate, hatchlings were allowed to swim of their own volition, without encouragement. The chamber was filled with seawater so that an air pocket of 2cm in height × 25 cm × 20 cm was left between the chamber lid and the water. Thus, the air volume during testing could be calculated following Withers (1977). Air was drawn from the chamber and passed through an Applied Electrochemistry O_2 Analyser S-3A (AEI Technologies, Pittsburgh, Pennsylvania USA) before being pumped into the atmosphere. Between turtles, we sanitized than tank and replaced the seawater with fresh, autoclaved seawater allowed to come to room temperature.

Hatchlings were randomly selected from each clutch for testing. All were weighed using an electronic balance or a Pesola$_{TM}$ scale. Leatherback, loggerhead and green hatchlings were tested during the frenzy (sample size ($N_{loggerheads}$)=21, mass ± se 18.39 ± 0.37g; N_{greens}=24, 24.72 ± 0.36g; $N_{leatherbacks}$=25, 44.89 ± 0.72g) and post-frenzy ($N_{loggerheads}$=28, 22.14 ± 1.06g; N_{greens}=33, 35.6 ± 1.48g; $N_{leatherbacks}$=25, 59.03 ± 2.58g) . Each hatchling was fitted with a Velcro strip using Vetbond as described above. Hatchlings were allowed to acclimatise for 30 min. Incurrent air was drawn continuously through a hole drilled in the chamber lid into the space between the chamber walls and the water inside the chamber. Air from inside the chamber was drawn through a second hole, passed through a water absorber (Drierite water absorbent, W.A. Hammond DRIERITE, Xenia, Ohio USA), a Mass Flow Controller (Sierra Side-Trak 840) and an Applied Electrochemistry Oxygen Analyser S-3A (AEI Technologies, Pittsburgh, Pennsylvania USA) before being pumped into the atmosphere. The O_2 analyser was calibrated before and after each trial with dry, CO_2 free air (22% N_2, 78% O_2 standard) and data was corrected for analyser drift and to STP.

Room temperature was recorded before each trial (23.61 ± 1.5°C). For resting and active metabolic rate, hatchlings were tested for 90 min and for crawling metabolic rate hatchlings were tested for 40 min.

Hatchling collection, testing and housing were conducted under Florida Sea Turtle Permit 073.

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Supplementary Table 5.1: Results from Tukey's pairwise comparisons of the metabolic rates of 'frenzied' green sea turtle hatchlings at various activity levels. Significant results are marked with *. RMR was measured in 103 hatchlings, CMR in 8 hatchlings, AMR in 14 hatchlings and MMR in 90 hatchlings.

	Crawling	***Maximal swimming***	***Resting***
Maximal swimming	z=-6.41, p<0.0001*		
Resting	z=4.591, P<0.0001*	z=31.105, p<0.0001*	
Routine swimming	z=-4.296, p=0.0001*	z=1.214, p=0.618	z=-10.721, p<0.0001*

Supplementary Table 5.2: Results from Tukey's pairwise comparisons of the resting metabolic rates of flatback (n=80,79), green (n=103,11), leatherback (n=8,6), loggerhead (n=3,15) and olive ridley turtles (n=74,70) during the frenzy and post-frenzy, respectively. Significant results are marked with *.

	Flatback	***Green***	***Leatherback***	***Loggerhead***
Frenzy				
Green	z=0.225, p=0.999			
Leatherback	z=-5.691, p<0.0001*	z=-5.833, p<0.0001*		
Loggerhead	z=0.13, p=0.999	z=0.074, p=1	z=2.132, p=0.207	
Olive ridley	z=4.011, p=0.0006*	z=3.863, p=0.0011*	z=7.65, p<0.0001*	z=1.388, p=0.635
Post-frenzy				
Green	z=-4.838, p<0.0001*			
Leatherback	z=-4.545, p=0.0001*	z=-0.282, p=0.999		
Loggerhead	z=-5.258, p<0.0001*	z=-0.133, p=0.999	z=0.18, p=0.999	
Olive ridley	z=0.121, p=1	z=4.767, p<0.0001*	z=4.508, p=0.0001*	z=5.157, p<0.0001*

Supplementary Table 5.3: Results from Tukey's pairwise comparisons of the crawling metabolic rates of green (n=8), leatherback (n=6) and loggerhead turtles (n=7) during the frenzy. Significant results are marked with *.

	Green	***Leatherback***
Leatherback	z=-2.002, p=0.265	
Loggerhead	z=-1.051, p=0.832	z=1.033, p=0.84

Supplementary Table 5.4: Results from Tukey's pairwise comparisons of the metabolic rates of green (n=14,23), leatherback (n=13,32) and loggerhead turtles (n=11,24) during 'frenzied' and post-frenzy routine swimming, respectively. Significant results are marked with *.

	Green	***Leatherback***
	Frenzy	
Leatherback	z=3.046, p=0.02*	
Loggerhead	z=1.504, p=0.56	z=-1.424, p=0.612
	Post-frenzy	
Leatherback	z=4.839, p<0.0001*	
Loggerhead	z=2.884, p=0.032*	z=-2.009, p=0.262

Supplementary Table 5.5: Results from Tukey's pairwise comparisons of the metabolic rates of flatback (n=79), green (n=90) and olive ridley turtles (n=71) during 'frenzied' maximal swimming. Significant results are marked with *.

	Flatback	***Green***
Green	z=-11.777, p<0.0001*	
Olive ridley	z=2.859, p=0.035*	z=14.19, p<0.0001*

Supplementary Table 5.6: Results from Tukey's pairwise comparisons of the aerobic scopes of flatback (n=79), green (n=90) and olive ridley turtles (n=71) during the frenzy. Significant results are marked with *.

	Flatback	Green
Green	t_{383}=-11.064, p<0.0001*	
Olive ridley	t_{383}=-4.788, p<0.0001*	t_{383}=5.811, p<0.0001*

Appendix III

Supplementary material for Chapter 6

Pipping green sea turtles

Photo taken by Christopher Gatto

Supplementary table 6.1: Tukey's HSD differences among age groups for each morphological measurement. Statistically significant differences are in bold.

	0 v 4 weeks	*0 v 12 weeks*	*0 v 24 weeks*	*4 v 12 weeks*	*4 v 24 weeks*	*12 v 24 weeks*
Head width (mm)	**t_{94}=-16.63, p<0.0001**	**$t_{121.8}$=-33.04, p<0.0001**	**$t_{137.5}$=-36.55, p<0.0001**	**$t_{121.8}$=-23.83, p<0.0001**	**$t_{137.5}$=-27.63, p<0.0001**	**$t_{135.4}$=-3.51, p=0.003**
SCL (mm)	**$t_{94.1}$=-24.59, p<0.0001**	**$t_{122.5}$=-47.9, p<0.0001**	**$t_{137.6}$=-53.19, p<0.0001**	**$t_{122.5}$=-34.26, p<0.0001**	**$t_{137.5}$=-39.93, p<0.0001**	**$t_{135.7}$=-5.17, p<0.0001**
SCW (mm)	**$t_{97.9}$=-24.44, p<0.0001**	**$t_{131.8}$=-4.63, p<0.0001**	**$t_{137.1}$=-45.94, p<0.0001**	**$t_{131.8}$=-28.69, p<0.0001**	**$t_{137.1}$=-32.08, p<0.0001**	**$t_{137.2}$=-2.77, P=0.045**
Flipper length (mm)	**$t_{93.4}$=-16.63, p<0.0001**	**$t_{119.3}$=-29.29, p<0.0001**	**$t_{138.6}$=-30.07, p<0.0001**	**$t_{119.3}$=-20.14, p<0.0001**	**$t_{138.6}$=-21.28, p<0.0001**	$t_{135.4}$=-1.49, P=0.44
Mass (g)	**t_{94}=-16.63, p<0.0001**	**$t_{121.8}$=-33.04, p<0.0001**	**$t_{137.5}$=-36.55, p<0.0001**	**$t_{121.8}$=-23.83, p<0.0001**	**$t_{137.5}$=-27.63, p<0.0001**	**$t_{135.4}$=-3.51, p=0.003**

Supplementary Table 6.2: Tukey's HSD differences among age groups for each swimming attribute. Statistically significant differences are in bold. Our linear mixed effect model found that most metrics increased with age. They did not detect a change in the proportion of time spent powerstroking.

	0 v 4 weeks	*0 v 12 weeks*	*0 v 24 weeks*	*4 v 12 weeks*	*4 v 24 weeks*	*12 v 24 weeks*
Mean swim thrust (N)	**$t_{98.2}$=-4.49, p=0.0001**	**$t_{131.9}$=-7.58, p<0.0001**	**$t_{137.9}$=-8.84, p<0.0001**	**$t_{131.9}$=-5.02, p<0.0001**	**$t_{137.9}$=-6.29, p<0.0001**	$t_{137.6}$=-1.01, p=0.74
Mean maximum thrust (N)	**$t_{96.5}$=-12.45, p<0.0001**	**$t_{128.9}$=-19.73, p<0.0001**	**$t_{138.1}$=-20.59, p<0.0001**	**$t_{128.9}$=-12.7, p<0.0001**	**$t_{138.1}$=-13.65, p<0.0001**	$t_{137.3}$=-0.87, p=0.82
Duration of power stroking bouts (s)	$t_{91.1}$=-2.25, p=0.12	$t_{108.8}$=2.28, p=0.11	$t_{137.6}$=2.06, p=0.17	**$t_{108.2}$=3.49, p=0.004**	**$t_{137.6}$=3.17, p=0.01**	$t_{131.7}$=-0.023, p=1
Power stroke frequency (str/min)	**$t_{98.3}$=6.17, p<0.0001**	$t_{131.9}$=-1.19, p=0.636	**$t_{138.1}$=-2.63, p=0.046**	**$t_{131.9}$=-4.71, p<0.0001**	**$t_{138.1}$=-6.127, p<0.0001**	$t_{137.8}$=-1.13, p=0.67

Work hard, party hard. The turtle squad at the 2019 ISTS symposium in Charleston, South Carolina.

Photos taken by (top) a passer-by and (bottom) Bill Matthews.