Ecophysiological responses of sea turtles to global change

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Details of collaboration:

Chapter 1: Christophe Eizaguirre (CE) and Emma Lockley (EL) conceived the experiment. EL and Perla Roman (PR) conducted the meta-analysis. EL conducted the statistical analysis. EL and PR created the mathematical projections. CE, EL and PR all contributed to the interpretation of the results. EL wrote the manuscript with comments and edits from CE.

Chapter 2: EL and CE conceived the experiment. Fieldwork was facilitated by Thomas Reischig (TR) and Turtle Foundation. Data collection was led by EL, and supported by CE, Sahmorie Cameron (SC) and Diego Fraile (DF). EL conducted all lab work and data analysis, and wrote the manuscript. EL and CE interpreted the results. CE provided feedback and edits on the manuscript. Gail Schofield and Chema Martín-Durán provided feedback.

Chapter 3: CE conceived and formed the long-term dataset included within this chapter. Data were collected by workers from the following organisations: Turtle Foundation, Projeto Biodiversidade, Fundação Maio Biodiversidade, Associação Projecto Vitó, Projeto Vito, National Institute for Fisheries Development and Biosfera. EL and CE collected nest and fitness data in Boavista along with SC and DF, while Kirsten Fairweather and Albert Taxonera collected this data in Sal. DNA extraction and species identification was completed by Rahmanullah Hayat. EL conducted all other analysis and all writing, with comments and edits of the manuscript by CE.

Chapter 4: Project was conceived by EL and CE. Fieldwork carried out by EL, CE, SC, DF and Emma Thomas. Laboratory work conducted by EL. All analysis and writing conducted by EL, with comments from CE.

In memory of my two wonderful nans,

Kathleen Pitchford and Jane Madeline Lockley

“There exists a presence in the ocean, seldom glimpsed in waking hours, best envisioned in your dreams. While you drift in sleep, turtles ride the curve of the deep, seeking their inspiration from the sky. From tranquil tropic bays or nightmare maelstroms hissing foam, they come unseen to share our air. Each sharp exhalation affirms, “Life yet endures.” Each inhaled gasp vows “Life will continue”. With each breath they declare to the stars and wild silence. By night and by light, sea turtles glide always, their parallel universe strangely alien, yet intertwining with ours.”

Carl Safina, Voyage of the Turtle: In Pursuit of the Earth’s Last Dinosaur

Abstract

The global effects of climate change are ubiquitous. Whether and how species respond to these changes will determine their populations' persistence. As long-lived marine ectotherms with temperature-dependent sex determination (TSD), sea turtles are highly vulnerable to global temperature changes. In this thesis, I explore responses to various environmental pressures on this taxon, and some of their underlying proximate mechanisms. In Chapter One, together with colleagues, I show that 35% of variation in the pivotal temperature (the incubation temperature producing 50:50 male/female offspring) among sea turtle populations can be explained by regional climate, suggesting local adaptation of this complex trait. Building various adaptive models projecting population demographics, I find neither speculated heritability nor plastic matching of the pivotal temperature to global warming would likely be sufficient to prevent the feminisation of most populations worldwide, if climate warming exceeds 2 °C. Chapter Two unveils a previously unknown mechanism in sea turtles, where maternally derived sex steroid hormones likely influence offspring sex ratios independently of temperature. This could be a possible mechanism to facilitate plasticity in the TSD response. Global change may also disrupt biotic interactions such as host-parasite dynamics. Chapter Three shows that the prevalence of leech parasites in the only significant rookery of loggerhead turtles in the eastern Atlantic has increased over the last decade. This increase has resulted in a possible size-specific trade-off, where the smallest infected turtles invest less in reproduction following a bet-hedging strategy, and the largest infected turtles terminally invest. Chapter Four provides some evidence that this immunity-reproduction trade-off is at least in part mediated by the sex steroid hormone oestradiol. Altogether, my work demonstrates how many elements of species' ecology and evolution are impacted by climate change. Understanding their response will contribute to more effective conservation measures.

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General Introduction

The current rate of species loss places the world in the sixth mass extinction event in geological history (Barnosky et al. 2011). The severity of observed habitat fragmentation and overexploitation is worsened by a rapidly changing climate, leading to "biological annihilation" (Ceballos et al. 2015; Ceballos et al. 2017). As climate change progresses and global temperatures rapidly increase, understanding how species interact with their environment has never been so important (Visser 2008; Hoffmann & Sgrò 2011; Stocker et al. 2013; Neukom et al. 2019).

Ectotherms are particularly at risk from increasing temperatures, as their distributions, physiology, and the evolution of key life-history traits are all tightly linked to their thermal environment (Pinsky et al. 2019). Marine ectotherms might be some of the most vulnerable species to global change, as they are frequently exposed to temperatures close to their upper thermal limits, compared to those in terrestrial habitats where thermal refugia are more accessible (Pinsky et al. 2019). Climate change may also interact with other anthropogenic activities to accelerate marine species' extinction risk (Chandra et al. 2012; Christiansen et al. 2014). For example, as temperate fish species follow warming sea surface temperatures pole-wards, they risk being taken as bycatch as they face new interactions with Arctic fisheries (Christiansen et al. 2014). Warm temperatures can further exacerbate the physiological effects of pollutants, such as artificial oestrogens on fish, which cause rapid changes in their breeding biology (Chandra et al. 2012). For marine species that breed on land, sea turtles and sea snakes for instance, sea level rise and coastal development may reduce availability of nesting habitats (Mazaris et al. 2009). Thus, for all species, but especially those vulnerable to these combined effects (and those populations that are already depleted), an understanding of whether and how they might respond, either through compensatory mechanisms or by rapid evolution, is crucial for efficient management and conservation

(Eizaguirre & Baltazar-Soares 2014).

Sea turtles are a taxon facing cumulative impacts from climate change and a variety of anthropogenic activities (McMahon & Hays 2006; Hawkes et al. 2007; Witt et al. 2010). They are affected by a multitude of human activities including coastal development (e.g. Kaska et al. 2013; Von Holle et al. 2019), bycatch (e.g. Senko et al. 2014; Fossette et al. 2014) and directed take (e.g. Tomillo et al. 2008; Senko et al. 2014). As a consequence, many populations are depleted, and are the subject of numerous conservation mitigation plans (Mortimer & Donnelly 2008; Hamann et al. 2010; Wallace et al. 2013; Casale & Tucker 2017). Sea turtles' breeding biology in particular is highly dependent on the thermal environment. In this thesis, together with coworkers, I examine how turtles' breeding biology interacts with environmental factors, to investigate populations' abilities to withstand and respond to changing global environments. With an initial focus on the temperature-dependent sex determination mechanism in this taxon, I first use adaptive and plastic response scenarios to revise predictions of how sea turtle population demographics will vary over the remainder of this century. I then take a close perspective on the effect of sea turtle physiology, in terms of maternal hormone transfer, on the sex determination process. As global change might disrupt host-parasite dynamics, I also explore the effects of a common leech parasite on feeding ecology and reproduction. I finish by identifying possible proximate hormonal mechanisms for observed fitness trade-offs in response to infection.

I.1. Climate Change

I.1.1 An overview

Anthropogenic activities increase the emission of greenhouse gases into the earth's atmosphere, and are driving a period of unprecedented change in global environments (Stocker et al. 2013; Neukom et al. 2019). Over the past three decades, the warming of

air and sea surface temperatures has become more pronounced (Stocker et al. 2013), causing ice sheets to melt (Hansen et al. 2016), and sea levels to rise (Hay et al. 2014). The addition of freshwater from the melting Greenland Ice Sheet weakens the Atlantic meridional overturning circulation, altering global ocean currents (Rahmstorf et al. 2015). Dissolved CO_2 modifies the chemistry of the oceans, reducing pH and causing ocean acidification and changes in biogeochemistry (Riebesell 2004; Doney et al. 2009; Lohbeck et al. 2012). Together, lower estimates of global risk for biodiversity suggest that approximately 18% of species worldwide are now "committed to extinction" (Thomas et al. 2004).

Responses to change are likely species-specific but are not independent of ecological interactions, resulting in the disruption of community interactions (Harrington et al. 1999). Trophic mismatch has already been documented in large studies of marine plankton communities, with timing of spring diatom blooms (likely dependent on photoperiod) remaining largely constant, while species that are dependent on temperature to regulate larval release have moved their seasonal cycles forwards in time (Edwards & Richardson 2004). Overall, species-specific responses to climate change will likely reduce ecosystem functioning, resilience and services (Scheffer et al. 2001; Pecl et al. 2017)

I.1.2 Species re-distributions

The changing global environment creates the possibility for new habitats to become suitable for species, at the same time that historically inhabited regions can no longer support populations. Range shifts have been described for dragonflies (Hickling et al. 2005), spiders (Krehenwinkel & Tautz 2013), fish (Perry et al. 2005), birds (Devictor et al. 2008) and mammals (Moritz & Agudo 2013), indicating that mobile taxa can follow their optimal thermal niche as it extends pole-ward (Hickling et al. 2006). Chen et al

(2011) reported a median shift in altitude of 11 metres per decade for 23 taxa, and a median shift in latitude of 16.9 kilometres over the same period, linking movements to temperature by showing re-distributions to be largest where temperature change was greatest. Moving pole-wards may not be sufficient to avoid the deleterious effects of rising temperatures, however. While a large survey of bird species in France showed that communities moved 91 kilometres northward across a 17 year period, they were required to relocate 237 kilometres north to remain in their thermal optimum (Devictor et al. 2008). Meta-analyses of range shifts show these are an order of magnitude faster in the oceans than on land, and in coastal environments, population shifts are successfully tracking changes in sea surface temperatures (Sorte et al. 2010; Poloczanska et al. 2013).

I.1.1.3 Shifts in phenology

Populations may also seek temporal, rather than spatial, refugia in the face of environmental change by changing their phenology (Edwards & Richardson 2004; Visser & Both 2005; Parmesan 2007). In plants and animals, earlier reproduction correlates with increasing spring temperatures recorded across Europe (Menzel et al. 2006). Three amphibian species in England have advanced their breeding season up to a month across a single decade (Beebee 1995). However, amphibians change their breeding phenology at twice the rate of birds, highlighting how such responses can be species/taxon specific (Parmesan 2007). Similarly, the phenological shift of a species will not be independent of the responses of communities, such as predator and prey interactions, which are notoriously asynchronous (Visser & Both 2005). Parmesan's (2007) meta-analysis showed that the advance in timing of many butterfly species' arrival at breeding grounds was three times faster than flowering herbs, exemplifying the risk of trophic mismatch. Such asynchrony can have ramifications for offspring

survival, as reported in great tits, *Parus major*, where a mismatch in timing between eggs hatching and the peak availability of their main food source, caterpillars, constrained the success rate and mass of fledgling birds (Visser et al. 2006).

I.1.4 Mechanisms for responses to climate change

Species responses to climate change may be adaptive, driven by directional selection - natural selection of advantageous phenotypes encoded by specific alleles, which will then propagate through populations (microevolution). Alternatively, responses may be linked to phenotypic plasticity, in which a single genotype expresses condition-dependent phenotypes which can be behavioural (as in the case of phenology) and/or physiological, in response to environmental cues (Hoffmann & Sgrò 2011). While the timeframe in which these two mechanisms act may overlap, they are slightly different. Microevolution requires that traits encoded by beneficial alleles be subject to selection across reproductive events. On the other hand plasticity in the expression of a trait can both happen within the lifespan of an individual and, in the case of trans-generational plasticity, between generations (Forsman 2015).

I.2. Responses to climate change: adaptation

Rapid climate change results in directional selection on, for example, traits that promote resilience to extreme conditions (Hoffmann & Sgrò 2011). Gienapp et al. (2008) proposed three criteria to assert evolutionary adaptation in response to climate change; i) trait selection must be observed or inferred; ii) there must be evidence that this trait is strongly linked to climate change, and iii) there must be a genetic basis to this trait. While these criteria may seem trivial, it is challenging to provide evidence that fulfils all of these benchmark requirements from natural populations. This is due to a lack of known genetic basis for given traits, and, as a result, phenotypic data alone are

frequently used to make evolutionary inferences (Gienapp et al. 2008). Some examples of evolved responses to changing environments do, however, exist (Figure I.1). Grant & Grant (2006) found evidence of selection on the beak size of the medium ground finch, *Geospiza fulginosa*, in response to drought conditions: beak size increased in response to drought conditions in 1977. In 2004, however, beak size decreased. The difference is attributed to the introduction of another *Geospiza* sp., which increased species-species competition. The genetic basis of beak shape is known and linked to the *ALX1* gene, which regulates craniofacial development (Lamichhaney et al. 2015). In the aquatic realm, a genetically-based increase in the thermal tolerance of *Daphnia* that hatched in lake sediments between 1960 and the 2000s has also been recorded, showing an evolved response to increasing temperatures (Geerts et al. 2015, Figure I.1). Finally, analysis of 32 years of genetic data from Alaskan pink salmon *Oncorhynchus gorbuscha* provided evidence of directional selection on the timing of migration: since 1993, there has been a three-fold decrease in the frequency of the "late migration" allele in the population, but minimal change in neutrally evolving loci (Kovach et al. 2012, Figure I.1).

Adaptive potential is defined by Eizaguirre & Baltazar-Soares (2014) as "the ability of populations/species to respond to selection by means of phenotypic or molecular changes". As rates of climate change increase, lags between trait and environment matching may increase, and might risk exceeding achievable rates of species' adaptive potential (Gienapp et al. 2008). For example, periods of intense directional selection can result in reduced genetic variation (Hoffmann & Sgrò 2011; Pauls et al. 2013). These effects are particularly pronounced in populations that are already suffering from low genetic diversity, either due to small effective population sizes, or barriers to gene flow such as habitat fragmentation (Gienapp et al. 2008; Willi & Hoffmann 2009; Hoffmann & Sgrò 2011; Pauls et al. 2013).

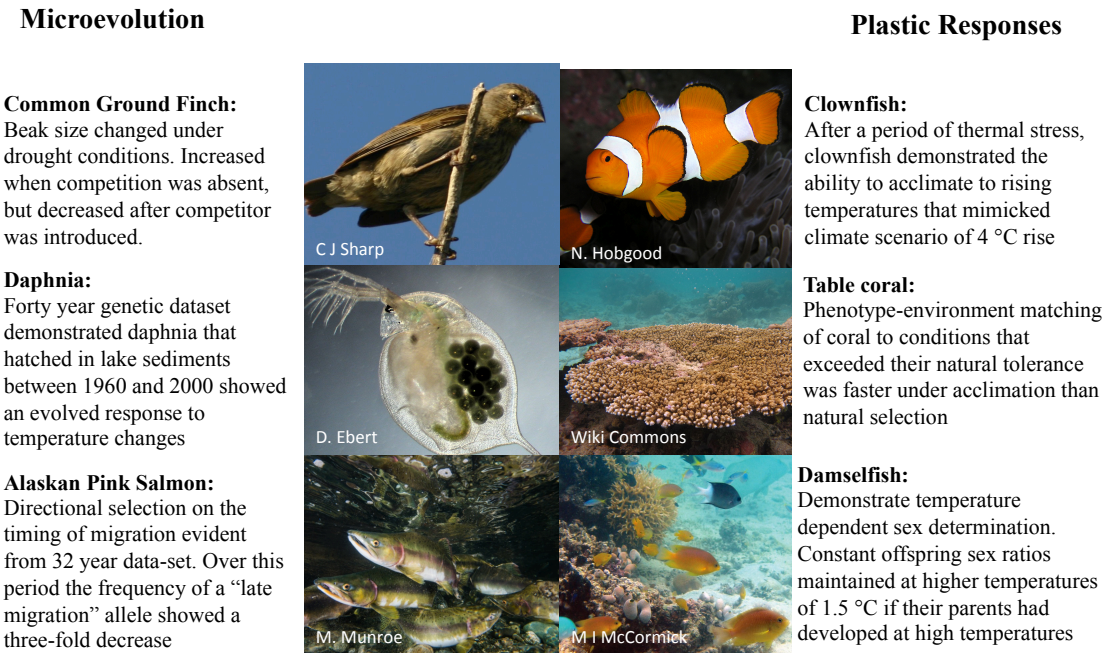

Figure I.1: Examples of microevolution and plasticity in response to climate change. A) Grant & Grant (2006) B) Geerts et al. (2015) C) Kovach et al. (2012) D) Madeira et al. (2016) E) Palumbi et al. (2014) F) Donelson et al. (2016)

The assumption that climate change will cause directional selection might also be too simplistic to inform about species' adaptive potential. Directional selection assumes a constant environment, whereas environmental heterogeneity and the predicted increased frequency of extreme events under climate change may result in fluctuating selection, in which the direction of selection varies over time. For example, in years of good environmental conditions, birth rates and survival in Soay sheep *Ovis aries* are high, and selection is relaxed. On the other hand, in years of harsh environmental conditions, heritability is constrained by the existing genetic variance within the population (Wilson et al. 2006). When adaptive potential is constrained, phenotypic plasticity may instead allow species to respond to their environment (Chevin et al. 2010).

I.3. Responses to climate change: plasticity

Non-genetic phenotypic plasticity is an alternative series of mechanisms to adaptation, by which organisms can match their phenotype to their environmental conditions (Gienapp et al. 2008; Chevin et al. 2010; Merilä & Hendry 2014). For example, juvenile daphnia *Daphnia pulex* develop morphological changes in response to chemical cues (small, jagged protrusions known as neckteeth) released by predatory phantom-midge larvae, *Chaoborus* sp (Tollrian 1995). Such context-dependent traits, and others, are underpinned by different mechanisms which include i) epigenetic regulation, for instance via whole genome DNA methylation, as observed in the coral *Pocillopora damicornis* when exposed to fluctuating pH conditions (Putnam et al. 2016)), ii) hormonal regulation of mechanisms in response to perceived environmental changes, as seen by ecorticotropin-releasing hormone activating accelerated development and metamorphosis of tadpoles during drought conditions (Denver 1997), and iii) behaviour, for example the selection of artificial (warmer) habitat by long-tailed skink *Eutropis longicaudata* that produces larger offspring with better survival (Huang & Pike 2011). Importantly, plastic responses may not always be beneficial, as hot temperatures during embryonic incubation cause reduced locomotor performance in leatherback sea turtles *Dermochelys coriacea* (Mickelson & Downie 2010). Decreases in the body size of salamander populations appear to be a consequence of a trade-off between metabolic rate and growth potential at high temperatures (Caruso et al. 2015).

In the specific context of phenotype-environment matching, evolutionary theory suggests that populations that demonstrate the highest plasticity in beneficial traits might have the greatest success in persisting with climate change (Meyers & Bull 2002). This is because if the cost of plasticity is low, it will reduce the range of conditions under which extinction is inevitable (Chevin et al. 2010; Sanford & Kelly 2011). Plasticity could thus maintain populations until adaptive evolution (and the

accumulation of random mutations) can improve the phenotype-environment match in the longer term (Lande 2009; Reusch 2014).

According to meta-analyses focusing on phenotypic change, phenotypic responses occurring under anthropogenic conditions appear to be more rapid than under natural contexts and, to date, have mostly been attributed to plasticity rather than genetic adaptation (Hendry et al. 2008; Gienapp et al. 2008; Merilä & Hendry 2014). Plastic responses frequently exist within-generation, and thus can exhibit a faster rate of phenotype-environment matching than genetic responses (Figure I.1). After a period of thermal stress, clownfish *Amphiprion ocellaris* were seen to successfully acclimate to ocean warming scenarios of up to 4 °C (Madeira et al. 2016). Relocating coral species to conditions that exceeded their thermal tolerances showed that acclimatisation accelerated environment-phenotype matching at a faster rate than natural selection (Palumbi et al. 2014).

In recent years, it has become clear that plasticity can also act across generations. Transgenerational plasticity (also known as parental effects) is the capacity of the phenotype of an offspring to be shaped by the environmental conditions of its parents through non-genetic mechanisms (Mousseau & Fox 1998; Badyaev & Uller 2009; Roth et al. 2018). To evolve, such plasticity must confer increased Darwinian fitness to parents or offspring (Marshall & Uller 2007). Evidence of trans-generational plasticity has mostly come from immune priming: if the parasitic environments of parents and progeny are likely to be similar, then it can be beneficial to transfer some additional, non-genetic, elements of immunity to the next generation (Marshall & Uller 2007; Kaufmann et al. 2014; Pigeault et al. 2016; Roth et al. 2018). Examples include the transfer of maternal antibodies to neonate harbour seals *Phoca vitulina* in response to Phocine Distemper Virus, which increases herd immunity and the time intervals between epidemics (Garnier et al. 2014). Cory's shearwater *Calonectris borealis*

vaccinated against Newcastle disease virus maintained antibodies across generations via egg-mediated trans-generational transfer (Ramos et al. 2014). While maternal transfer of antibodies can be an effective mechanism to boost offspring immunity, molecular processes can also facilitate this. For example, authors successfully attributed epigenetic processes to genes expressed when exposing one-week-old pipefish *Sygnathus typhle* offspring to an immune challenge that their grandparents were previously exposed to (Beemelmanns & Roth 2017).

With regards to climate change, transgenerational plasticity may be common, yet difficult to predict (Donelson et al. 2018). Transgenerational plasticity can partly alleviate the pressures of ocean acidification on copepod *Pseudocalanus acuspes* fecundity, with offspring able to maintain higher levels of reproduction than expected under extremely low pH conditions (Thor & Dupont 2015). Tropical damselfish *Acanthochromis polyacanthus*, a species with temperature-dependent sex determination, maintain similar offspring sex ratios at temperatures 1.5 °C above present day averages if their parents had developed under high temperature conditions (Donelson & Munday 2015). However, at higher temperatures, this effect was lost (Donelson & Munday 2015).

It is important to note that plastic responses alone may not be sufficient to mitigate the challenges of climate change (Visser 2008; Gienapp et al. 2008). If the correlations between environmental cues and plastic responses become disrupted with the changing environment, they will no longer be beneficial (Visser 2008; Gienapp et al. 2008). Plastic responses may evolve in a genotype x environment interaction, and hypotheses suggest that plasticity will facilitate tolerance to environmental conditions that fall within a natural range of values (Chevin & Hoffmann 2017). Yet, if this range is exceeded, then genetic selection might be the only way that population phenotypes can respond over time to extreme changes in environments (Chevin & Hoffmann 2017).

However, Chevin & Hoffmann (2017) also argue that an initial lack of genetic variation might have constrained plasticity from evolving to tolerate extremes, and this same lack of genetic variation will therefore limit the ability for evolution to act where plasticity has failed.

I.4. Plasticity: a key mechanistic role for hormones

I.4.1 Hormone synthesis and action

The endocrine and neuroendocrine pathways are two proximate mechanisms by which plasticity can occur. Hormones act as signalling molecules known as "biological agents of coordination" (John-Alder et al. 2009). They have varied chemical structure and form a central mechanistic link between environmental perception and trait expression (Wingfield 2008). They are responsible for regulating both temporary and permanent changes in physiology (e.g. Emlen & Nijhout 1999), life history (e.g. Hau et al. 2010) and behaviour (e.g. Owen et al. 2014). Endocrine pathways begin at a site, such as a gland, at which hormone synthesis and secretion occur in response to internal (e.g. the production of corticosterone in the adrenal glands is stimulated by adrenocorticotropic hormone (Handa et al. 1994)) or external cues (e.g. increasing photoperiod stimulates gonadotropin-releasing hormone in birds (Dawson et al. 2001)). Transport of hormones to target tissues generally occurs through the circulatory system (Norris 1997). After arriving at the target tissue, hormones bind to receptor proteins and trigger cascades that can result in, for example, the transcription of specific genes (Ketterson & Nolan Jr 1999).

To date, research in endocrinology has been unrepresentative in terms of taxa and sex, with the majority of findings produced from studies on breeding male birds and fish (McCormick 2001; Kempenaers et al. 2008a; Guiguen et al. 2010; Cornelius et al. 2012; Jennifer C. Owen et al. 2014; Rosvall et al. 2016; Cooper et al. 2019; Tannenbaum et al.

2019). However, the evolutionary significance of the endocrine system in regulating optimised environmental responses means we must instead begin to direct our attention towards the non-model, wild species that are most vulnerable to environmental change.

I.4.2 Hormones as regulators of behaviour

Hormones regulate many aspects of behaviour (Hau et al. 2010; Hau & Goymann 2015). For instance, hormones play a functional role in dominance hierarchies in baboons (Gesquiere et al. 2011), in coordinating parental care in burying beetles (Engel et al. 2016), and enhancing memory and learning in lizards (Korol & Pisani 2015). Migratory behaviour, for example, is regulated by a set of complex hormonal cascades, and has been widely studied (Dingle 1996; Dawson et al. 2001; Dawson 2008). Removal of gonads during early winter in sparrows eliminates pre-migratory fattening and reduces 'zugenruhe' (migratory restlessness) (Wingfield et al. 1990), while experimental testosterone implants advanced the onset of zugenruhe in gray catbirds *Dumetella carolinensis* (Owen et al. 2014). Elevated levels of testosterone also increase the accumulation of muscle in the shoulders and pectoral muscles of sea turtles, crucial for their long distance feeding migrations (Jessop et al. 2004). The energetic demands of migration may result in carry-over effects for subsequent activities - "stressful" migrations in female black-browed albatross *Thalassarch melanophris* result in high levels of testosterone, which were linked to deferred breeding decisions and reduced reproductive success (Crossin et al. 2012).

I.4.3 Hormonal pleiotropy mediates biological trade-offs

Circulating hormones can concurrently target multiple tissues and simultaneously control an entire suite of traits, in a process known as hormonal pleiotropy (Ketterson & Nolan Jr 1992). Trade-offs might then be observed between traits, with the expression

of both beneficial (possibly adaptive) and unfavourable phenotypes at the same time. For instance, in male vertebrates, up-regulation of testosterone prior to mating is positively associated with traits related to secondary sexual selection, including ornamentation (Verhulst et al. 1999), aggression (Marler & Moore 1988), and mating behaviour (McGlothlin et al. 2007). Yet, testosterone impairs cell mediated immunity by inhibiting transcriptional factors that facilitate the production of anti-parasitic cytokines (McKay & Cidlowski 1999). High testosterone in individuals often results in immunosuppression (but see Peters 2000; Desprat et al. 2015) that can lead to increased susceptibility to parasite infection, a relationship that appears to be particularly strong in reptiles (Klukowski & Nelson 2001; Roberts et al. 2004; Pollock et al. 2012; Cornelius et al. 2014). This trade-off between sexual traits and immunity forms the basis of the Immunocompetence Handicap Hypothesis, which proposes that pleiotropy facilitates honest signalling of male condition through secondary sexual characteristics, resulting in the coevolution of female mate choice (Zahavi 1975; Hamilton & Zuk 1982; Milinski & Bakker 1990). Only males of high genetic quality can maintain the elevated concentrations of testosterone required for elaborate ornamentation (Folstad & Karter 1992; Foo et al. 2016).

The main hormone associated with reproduction in female vertebrates is oestradiol. Circulating concentrations of this hormone peak before or during the onset of vitellogenesis, (Kummrow et al. 2010; Currylow et al. 2013; Blas et al. 2010; Gramapurohit & Radder 2013). Oestradiol plays a crucial role in regulating this process, during which yolk pre-cursor proteins called vitellogenins are produced in the liver (Ho et al. 1982). Vitellogenesis is energetically costly – maternal metabolism in snakes rises by 30% during this period, which is significantly greater than during pregnancy (Dyke & Beaupre 2011). Consequently, maternal body fat reserves are also mobilised (Bonnet et al. 1994; Hamann et al. 2002).

Similar to testosterone, oestradiol also suppresses cell-mediated immunity (Foo et al. 2016). However, this hormone enhances humeral immune responses (the up-regulation of antibodies unique to specific antigens) (Klein 2004). A recent meta-analysis of experimental studies demonstrated a medium-to-large effect of oestradiol on reducing parasite loads, and an enhancing effect on anti-inflammatory cytokine levels (Foo et al. 2016). In the field, the link between oestradiol and parasite load remains more equivocal, for instance there was no relationship between this hormone and parasite load in wild roach (Vainikka et al. 2004), but oestradiol shows immunosuppressive effect on phagocytic cells in common carp (Watanuki et al. 2002). The fact that oestradiol suppresses cell-mediated immune responses while enhancing humeral responses may be due to differential costs (Foo et al. 2016). Cell-mediated immune responses have greater energetic requirements than humeral-mediated responses (Janeway et al. 1999). Lee (2006) suggested that if females have a low-cost humeral immune response, it enables them to re-direct resources to reproduction while still maintaining a healthy immune system.

I.4.4 The Role of Hormones in Transgenerational Plasticity

Maternal hormone exposure during embryonic development can also influence individuals' offspring survival and fitness (Groothuis et al. 2005). Experimentally varying perceived population density by manipulating territorial vocalisations of red squirrels, *Tamiasciurus hudsonicus*, caused females to express higher levels of glucocorticoid levels (Dantzer & Swanson 2017). Females consequently produced offspring with faster growth rates, an adaptive response to the perception of elevated competition (Dantzer & Swanson 2017). In oviparous species, a principal conduit of hormones from mother to offspring is via the egg-yolk (Schwabl 1993; Radder 2007). For those species that show no maternal care, egg provisioning is an especially

important route in which reproductively active females can manipulate the environment of developing embryos. Testosterone, for example, affects growth rates of reptiles, but its precise role remains elusive: Elevated levels of egg testosterone increase growth rates in the dragon lizard *Ctenophorus fordi* (Uller et al. 2007) and common lizard *Zootoca vivipara* (Uller & Olsson 2003). However, the opposite effect is seen in common lizard offspring when exposed to tick parasites, apparently indicative of the pleiotropic function of testosterone in immune function (Uller & Olsson 2003).

I.5. Vulnerable biological mechanisms: temperature-dependent sex determination

Examining how populations respond to climate warming, either via genetic adaptation or phenotypic plasticity, is most powerful when considering the key biological mechanisms that are directly impacted by temperature. Temperature-dependent sex determination (TSD) is one such mechanism. Briefly, TSD is a form of environmental sex determination. TSD species do not have sex chromosomes, but instead the temperature during a thermosensitive period of development determines whether an individual develops as male or female. TSD was first reported in the common agama lizard, *Agama agama* in 1966 (Charnier 1966). Since then, it has been confirmed as the primary sex determination mechanism of several reptile lineages, including the tuatara, crocodilians and turtles (Janzen & Paukstis 1991; Cree et al. 1995). TSD is likely to have a single ancient origin, approximately 300 million years ago (Janzen & Krenz 2004). Different patterns of TSD exist (Figure I.2). In Type Ia TSD, males develop at lower temperatures, with females produced at warmer temperatures (e.g. the painted turtle, *Chrysemys picta* (Bull & Vogt 1979)). In Type 1b, this pattern is reversed, and males are produced at warm temperatures (e.g. the tuatara, *Sphenodon punctatus* (Cree et al. 1995)). Finally, Type II TSD species produce males at intermediate temperatures, and females at both hot and cold extremes (e.g. the American alligator, *Alligator*

mississippiensis (Ferguson & Joanen 1983)). The TSD thermal response curve is described by i) a pivotal temperature, at which there is an equal likelihood of an embryo developing as male or female, and ii) the range of temperatures at which either male or female offspring may be produced, known as the transitional range of temperatures (Figure I.2, Mrosovsky & Pieau 1991).

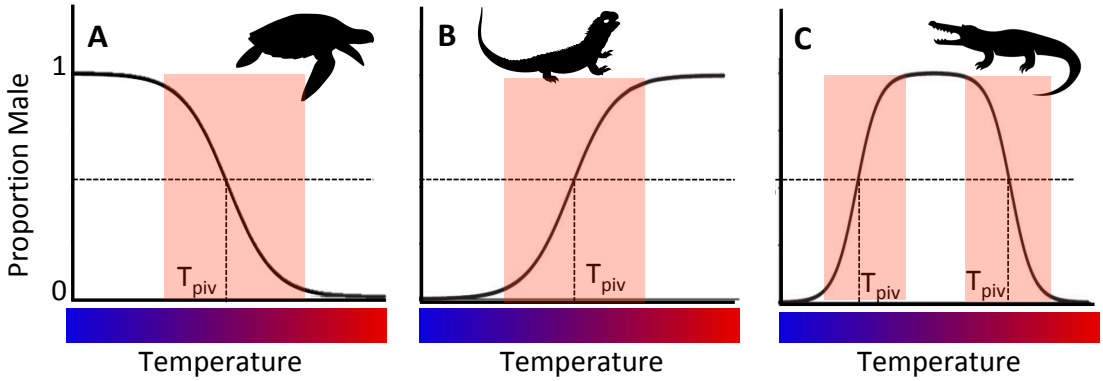

Figure I.2: The three patterns of temperature-dependent sex determination: A) Type 1A, as seen in sea turtles; B) Type 1B, as seen in tuatara; C) Type 2, as seen in crocodilians. Pivotal temperature (T_{piv}) is the temperature at which even proportions of males and females are produced. Red denotes the transitional range of temperatures, where either sex can be produced.

Current climate change challenges the survival of TSD species (Schwanz & Janzen 2008; Hulin et al. 2009; Telemeco et al. 2013). As temperatures rise, offspring sex ratios may become highly biased in favour of a single sex. For instance, as male offspring are produced at high temperatures in the two extant tuatara species, *Sphenodon punctatus* and *S. guntheri*, by the year 2085 all offspring are predicted to develop as males (Mitchell et al. 2010). This will eventually drive these already severely endangered species to extinction. TSD species have, however, survived historical episodes of extreme thermal variation, for example the rapid thermal change

recorded in the mid- to late- Cretaceous period (Huber et al. 2002; Hu et al. 2012), or the widely studied Cretaceous-Paleogene mass extinction period (Markwick 1998; Longrich et al. 2012; Puertolas-Pascual et al. 2016). This longevity in the face of thermal variation suggests TSD species may have evolved mechanisms that enable them to respond to climate change.

The enigmatic evolutionary significance of TSD is best explained by two hypotheses; the Charnov-Bull model of differential fitness, and that of phylogenetic inertia (reviewed by Shine (1999) and Janzen & Phillips (2006)). The Charnov-Bull model suggests sex specific benefits exist under sex specific thermal environments (Charnov & Bull 1977). Empirical support for this theory was demonstrated in the Jacky Dragon, *Amphibolurus muricatus*, by Warner & Shine (2008). Eggs from this agamid lizard were incubated at a range of temperatures, and half of the eggs were treated with an aromatase inhibitor (Box 1) that caused embryos to develop as male, regardless of thermal environment. After raising these individuals in field enclosures for 3.5 years, they found that lifetime reproductive success was greater for those males that were incubated at natural male-producing temperatures. There have however, been conflicting results among experiments testing the Charnov-Bull model (Janzen & Phillips 2006). For instance, in diamondback terrapin *Malaclemys terrapin,* egg mass positively correlates with hatchling size, which in turn has sex specific benefits for female offspring, by decreasing their growth time to minimum reproductive size (Roosenburg 1996). Roosenburg et al. (1996) found maternal nest choice favoured depositing clutches of large eggs in warm, female producing conditions, supporting the Charnov-Bull theory. However, the same relationship was absent in *C. picta* (Morjan & Janzen 2003). As such, Janzen & Phillips (2006) suggest that caution is needed when presenting the Charnov-Bull model as a catch-all explanation for TSD in reptiles.

The contradictory evidence for the Charnov-Bull model may be attributed to the ancient origin of TSD in reptiles, which suggests that the adaptive significance of TSD may no longer be detectable in all species, and that this trait instead exists as a product of phylogenetic inertia (Janzen & Krenz 2004; Janzen & Phillips 2006). After phylogenetically reconstructing the evolution of sex determination in squamata using over 400 species, Pokorná & Kratochvil (2009) found many examples of transitions from TSD to genetic sex determination, but no cases where this direction was reversed. Sex chromosomes may evolve when genes biasing sex determination towards male or female are coupled with genes that provide a selective advantage to that sex. As the association between these genes strengthens and rates of recombination decrease, sex chromosomes evolve (Muralidhar & Veller 2018).

The description of molecular pathways that instigate gonad differentiation in TSD species remains incomplete. Many mammalian genes involved in sex determination processes (e.g. *SF1*, *Sox9*, *AMH* and *Dmrt1*) exist in the genomes of TSD reptile species (e.g. Yao & Capel 2005). However, their regulation occurs after sex differentiation of the embryo has occurred (Lance 2009). The wide thermosensitive period of TSD species suggests that the process of differentiation is not a response to a rapid trigger, but instead a process of accumulation/suppression of a gene product (Lance 2009). The recent finding of Ge et al. (2018), using the model red-eared slider turtle *Trachemys scripta,* is possibly the first example of a causal link between molecular processes and sex determination. The authors demonstrated how the epigenetic regulator *Kdm6b* demethylates the histone H3 lysine 27 (*H3K27*) at the *Dmrt1* promoter region, in a process that results in male sex determination. However, *Kdm6b* is not in itself responsive to temperature, and as such the fundamental thermal trigger of this pathway remains unknown.

While temperature is the primary determinant of gonad differentiation in TSD species, other environmental conditions, such as precipitation and humidity, have also been found to influence this process (Houghton et al. 2007; Wyneken & Lolavar 2015). For example Wyneken and Lolavar (2015) suggest that more male hatchlings are produced than expected under high temperatures when moisture conditions of the sand are also elevated. There is also a vast literature that describes the effect of sex steroid hormones on the TSD proess (Elf 2003). For example, *in vitro* manipulation experiments show that the exogenous application of oestradiol produces female offspring at male-producing temperatures (Crews et al. 1989; Crews et al. 1991; Wibbels et al. 1991b). Oestradiol was also shown to regulate *Kdm6b* in the same manner as temperature in Ge et al. (2018). On the other hand, the application of aromatase inhibitors, which prevent the synthesis of oestradiol from its precursor androgen, testosterone, often results in male offspring (Wibbels & Crews 1994; Rhen & Lang 1995). These responses are however not always that predictable - negative results have been reported, along with incidences where exogenous application of oestradiol has produced male offspring (Janes et al. 2007; Warner et al. 2014). This may be a product of species-specific responses, or the difficulty of ensuring that exogenous hormone application represents biologically meaningful concentrations.

The role of hormones in sex determination in TSD species is also visible in a selection of field studies conducted on nesting painted turtles. When clutches are incubated at constant temperatures, variation in endogenous oestradiol and testosterone concentrations in the yolk correlate with the ultimate clutch sex ratio (Bowden et al. 2000). The quantities of these hormones transferred to clutches vary throughout a season, essentially modifying the thermal response of a nest (Carter et al. 2017). Therefore, while the primary driver of TSD is evidently temperature itself, maternal hormone transfer is likely to modify the temperature at which responses occur.

Variables that act upon the TSD mechanism alongside temperature may be important mechanisms to respond to climate change.

Box 1: Aromatase

Aromatase is an enzyme encoded by the *CYP19A1* gene (Strauss & FitzGerald 2018), and is part of the cytochrome P450 superfamily. While a single gene encodes human aromatase, in fish and reptiles there are two aromatase isomorphs, encoded by the *Cyp19a1* and *Cyp19b1* genes, expressed in the gonads and brain respectively (Boon & Simpson 2012). Its role is to convert androgens such as testosterone into oestradiol (Boon & Simpson 2012). Up-regulation of this enzyme in the gonad is required for ovarian differentiation in fish (Guiguen et al. 2010), birds (Smith et al. 1997) and reptiles (Jeyasuria & Place 1998), but not mammals, where the deletion of the aromatase enzyme does not prevent ovaries from developing (Fisher et al. 1998). In zebrafish, knockout of the *Cyp19a1* gene in developing embryos leads to male offspring (Lau et al. 2016).

The function of aromatase in gonad development means that this enzyme is thought to be a potential mediator of temperature-dependent sex determination. In European sea bass *Dicentrarchus labrax*, exposure to male producing temperatures results in methylation of the *Cyp19a1* gene and lower aromatase expression (Navarro-Martín et al. 2011). Similar methylation responses to temperature are also seen in red-eared slider turtles *Trachemys scripta* (Matsumoto et al. 2016). In artificial incubation experiments, exogenous application of aromatase inhibitors have repeatedly resulted in male embryos developing at female producing temperatures (e.g. Crews & Bergeron 1994; Warner & Shine 2008).

I.6. Sea Turtles

Sea turtles are particularly vulnerable to environmental change (Hamann et al. 2010;

Witt et al. 2010; Nelms et al. 2016). Seven extant sea turtle species are recognized and six of them are listed as "vulnerable" or higher on the IUCN red list (Figure I.3). This is the direct consequence of anthropogenic pressures such as harvest for food (Tomillo et al. 2008; Senko et al. 2014) and trade (Foran & Rays 2016), as well as indirect effects of fisheries by-catch (Senko et al. 2014; Fossette et al. 2014; Casale et al. 2015), pollution (Witherington et al. 2012; Schuyler et al. 2013) and coastal development (Harewood & Horrocks 2008; Kaska et al. 2013). In addition, sea turtle demographics and physiology are tightly linked to temperature, and so climate change will impact all stages of sea turtle life history (Hawkes et al. 2009; Witt et al. 2010; Pikesley et al. 2015) (Figure I.4). With most sea turtle species already considered vulnerable to extinction, there is a time limited imperative to understand the impacts of climate and the wider environment on this taxon (Hamann, M.H. Godfrey, et al. 2010).

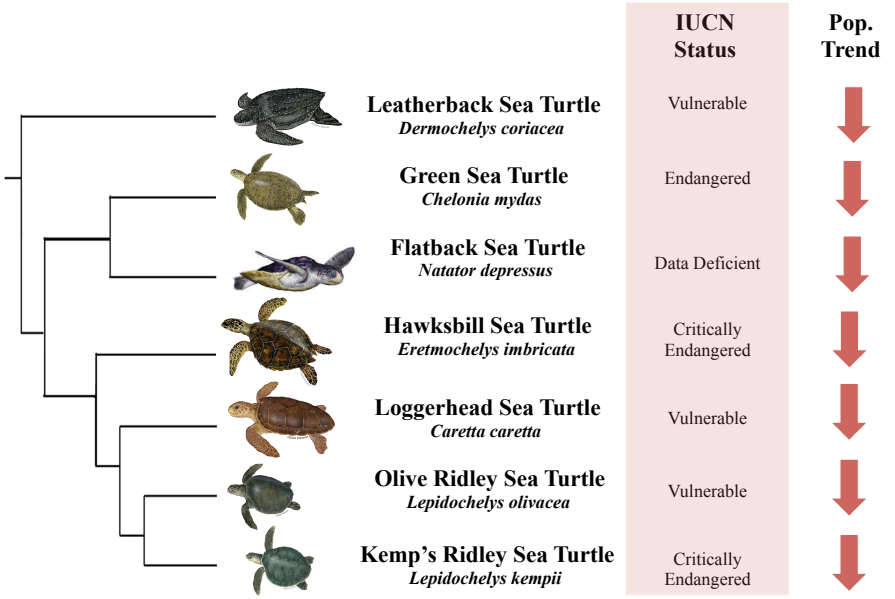

Figure I.3: Phylogeny of the seven extant sea turtle species (Naro-Maciel et al. 2008), along with their IUCN status and current global population trend status (IUCN Subcommittee 1996; Seminoff 2004; Mortimer & Donnelly 2008; Abreu-Grobois & Plotkin 2008; Wallace et al. 2013; Casale & Tucker 2017; Wibbels & Bevan 2019).

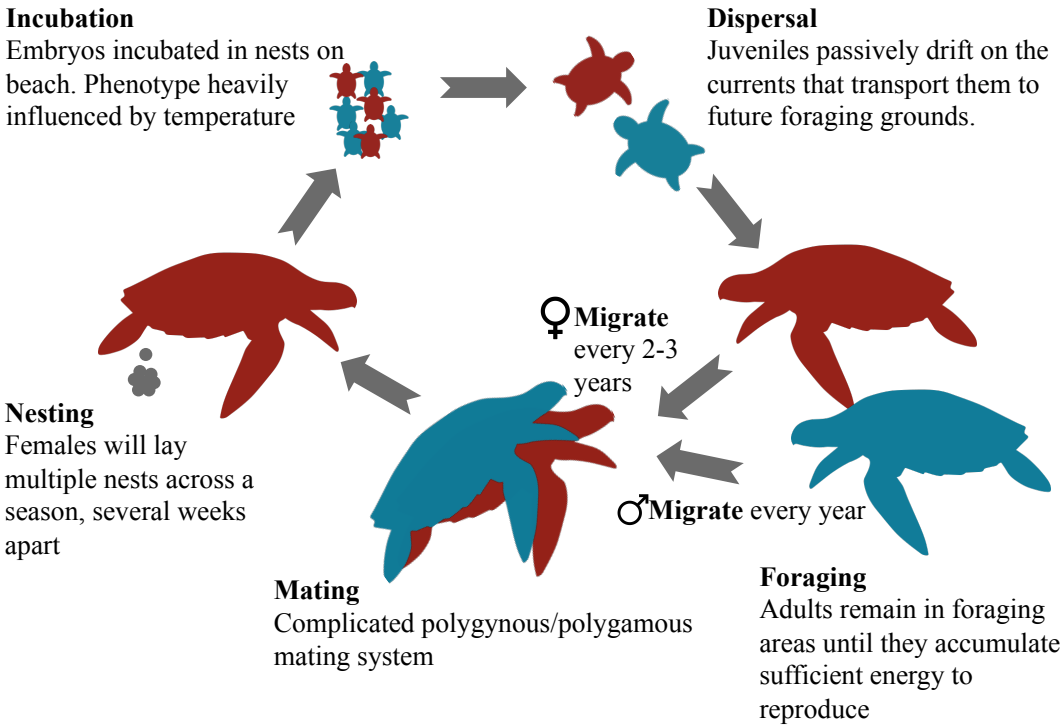

Figure I.4: Sea turtle lifecycle

I.6.1 The Incubation Environment

Sea turtles are Type 1a TSD species (Yntema & Mrosovsky 1982) and, as such, thermal projections across the coming century suggest that offspring sex ratios will become increasingly feminised (Yntema & Mrosovsky 1982; Hawkes et al. 2007; Hawkes et al. 2009; Witt et al. 2010; Laloë et al. 2014; Tanner et al. 2019). Population-specific estimates suggest that, by the end of the century, up to 99% of offspring produced in the loggerhead sea turtle (*Caretta caretta)* population of Cape Verde will be female (Laloë et al. 2014). The effects of rising temperatures are not restricted to sex determination, however. High incubation temperatures, for example, are thought to reduce offspring swimming performance in loggerhead and green turtles, *Chelonia mydas* (Kobayashi et al. 2017; Booth & Evans 2011), reduce crawling speeds in olive ridley turtle, *Lepidochelys olivacea,* (Maulany et al. 2012) and increase embryonic mortality rates in loggerhead turtles (Kobayashi et al. 2017). Yet, there is some evidence of local

adaptation of turtle embryos to high heat conditions. Green sea turtle offspring from dark sand (hot) beaches on Ascension Island grew faster and had higher levels of survival than those from nearby white sand (cool) beaches when exposed to hot artificial incubation environments (Weber et al. 2012). Incubating flatback turtle *Natator depressus* eggs at tropical latitudes in Australia have high levels of tolerance to exposure to prolonged warming periods of up to 35 °C, despite these temperatures being associated with reduced developmental success in olive ridley turtles in Indonesia (Maulany et al. 2012; Howard et al. 2015). Indeed, 35 °C is frequently cited as the lethal temperature for sea turtle embryos, but this is unlikely to be the case for all populations (Ackerman 1997).

With rising temperatures there is evidence that nesting seasons are starting earlier and are more protracted for loggerhead turtles nesting in North Carolina and Greece (Weishampel et al. 2004; Hawkes et al. 2007; Mazaris et al. 2013; Patel et al. 2016). From a global meta-analysis perspective, there is a significant negative relationship between the dates of first nesting for loggerhead sea turtles across their nesting distribution, and the sea surface temperature at the beginning of the nesting season (Mazaris et al. 2013). While nesting phenology has been correlated with increasing temperatures, other aspects of maternal behaviour appear more conserved. Although considerable spatiotemporal variation in temperature was recorded over 26 years at a loggerhead turtle nesting ground in North Carolina, very little variation was predicted in the sex ratios of clutches produced by the same individual, suggesting that nest-site selection, and behavioural plasticity, may be constrained in this population (Reneker & Kamel 2016).

Although much research focuses on responses linked to nesting behaviours, little attention has been given to physiological mechanisms. This neglect is unwarranted given mechanisms, such as maternal hormone transfer, may form adaptive responses to

climate change. *In vitro* oestradiol treatment of olive ridley turtle eggs at male producing temperatures can feminise gonads (Merchant-Larios et al. 1997), limit gonad growth (Diaz-Hernandez et al. 2014) and regulate cell proliferation (Díaz-Hernández et al. 2017), showing some alternative mechanisms of sex determination. There is, however, a lack of field studies that translate these in vitro results into an ecologically relevant context. This knowledge gap stems from the difficulty to determine the sex of hatchlings non-lethally. As many sea turtle populations are listed as threatened under the IUCN red list, euthanising hatchlings is often unfeasible.

I.6.2 Dispersal

Upon emergence from the nest, hatchlings must rapidly disperse beyond the continental shelf to avoid predation on the beach and near-shore waters, and reach offshore currents that transport them to future foraging grounds (Wyneken & Salmon 1992; Putman et al. 2012; Scott et al. 2014A; Scott et al. 2014B). Dispersal capacity correlates with traits such as size and, notably, swimming ability (Booth & Evans 2011; Scott et al. 2014A). Noteworthy, both of these traits are affected by incubation environment: i) in a split-clutch experimental design, offspring from cool nests were larger than those from warm nests (Booth et al. 2013) and ii) hatchlings from nests below 26 °C had better swim thrust (a combination of the time spent swimming, the flipper stroke rate and the peak thrust through the water) than those originating from nests that incubated above 30 °C (Booth & Evans (2011)).

When they reach offshore currents, hatchlings were historically thought to passively drift, adopting a low-energy feeding strategy (Witherington 2002). Recently, with the development of technology that allows us to track relatively small (14.1 – 21.9 cm) juvenile sea turtles via satellite telemetry, it has been shown that individuals use directed swimming in some species- and location- specific situations (Mansfield et al.

2009; Putman & Mansfield 2015; Briscoe et al. 2016). Nevertheless, lagrangian drift modelling scenarios have facilitated discoveries that the migratory routes of adult turtles are strongly linked to those currents that they experienced as hatchlings, thought to be an adaptation to ensure that turtles locate suitable foraging locations (e.g. Hays et al. 2010; Scott et al. 2014B).

I.6.3 Foraging

Sea turtle species have a variety of feeding strategies, from leatherback turtles, *Dermochelys coriacea*, which are obligate gelatinous planktivores (Houghton et al. 2006), and herbivorous green turtles (Bjorndal 1980), to opportunistic carnivore loggerhead turtles, which target a range of pelagic and oceanic prey species (Frick et al. 2009, Cameron et al. 2019). Some populations of turtles also demonstrate a dichotomous feeding strategy (Hatase et al. 2002; Hawkes et al. 2006; Hawkes et al. 2007). Early studies assumed that such dichotomies were linked to an ontogenetic shift between oceanic and neritic habitats (Hatase et al. 2002; Hawkes et al. 2006) and that oceanic turtles, which are the most common in some populations, paradoxically utilise suboptimal feeding grounds (e.g. Cape Verde, Eder et al. 2012). However, results from recent, large-scale studies show that there is no size difference between oceanic and neritic turtles, which challenges the theory of an ontogenetic shift (Cameron et al. 2019). Using stable isotope analysis of scute layers in loggerhead sea turtles, Cardona et al. (2017) showed that shifts between oceanic and neritic feeding by individuals likely do exist but are relatively rare – yet they reinforce the hypothesis that in some populations the distribution of turtles between neritic and ocean habitats is probably not the result of ontogenetic shift. Instead, there is growing evidence of a link between hatchling dispersal and adult migration routes (Hays ey al. 2010; Scott et al. 2014B), whereby foraging strategies are likely imprinted at an early stage of development and

adults show high site-fidelity to their foraging grounds (Broderick et al. 2007; Schofield et al. 2010).

I.6.4 Mating

Upon reaching sexual maturity, sea turtles show a high degree of natal philopatry, and migrate back to the rookery from which they originate, often with extreme fidelity (Meylan et al. 1990; Bowen & Karl 2007; Lee et al. 2007). This high fidelity limits gene flow among geographically distinct rookeries and nesting sites, and increases the potential for local adaptation. Stiebens et al. (2013) showed that high site fidelity of Cape Verde loggerhead sea turtle population results in multiple genetically distinct sub-populations. These sub-populations support different assemblages of alleles associated with the major histocompatibility complex (MHC are genes of the adaptive immune system of jawed vertebrates associated with parasite resistance), which provide adaptive potential to the overall population (Eizaguirre & Baltazar-Soares 2014).

Female turtles return to nest at approximately two- to three- year intervals, while males return more frequently, or even remain resident and do not migrate to feed (Schofield et al. 2010; Arendt et al. 2012; Hays et al. 2010). This difference in re-migration interval results in more frequent mating opportunities for males, and thus a different operational sex ratio (the ratio of sexually active males and females at a given time) than that of adult sex ratios (Hays et al. 2014). Operational sex ratios are less female-biased, and provide a possible buffer against highly feminised offspring sex ratios as they reach maturity(Wright et al. 2012; Hays et al. 2014) Yet, a predicted reduction in the total number of adult males in a population will still reduce genetic diversity (Frankham 2005), and increase potential levels of inbreeding and genetic drift (Hedrick & Kalinowski 2000), ultimately reducing population fitness and adaptive potential (Reed & Frankham 2003).

Interestingly, sea turtles are polygynadrous, and females may produce clutches of eggs fathered by multiple males while males also mate with multiple females (Kichler et al. 1999; Crim et al. 2002; Lee & Hays 2004; Wright et al. 2012; Lee et al. 2018). Lee et al (2018) found that multiple paternity was positively associated with population density within a mating area, and maternal body size and experience have also been positively correlated with the number of sires of a clutch (Zbinden et al. 2007; Lasala et al. 2013; Howe et al. 2018).

6.5 Study Population

Experimental studies in this thesis focused on nesting loggerhead turtles *Caretta caretta* as a model species. Loggerhead sea turtles have the widest nesting distribution of all extant sea turtle species, making them an excellent system for investigating patterns of local adaptation. Our specific study population is located in the islands of Cape Verde (Figure I.5). This is home to the third largest nesting aggregation worldwide (Marco et al. 2012). Historically, this population has been under considerable pressure from poaching activity on the islands, but thanks to the actions of NGOs, the number of sea turtles taken each year is reducing (Marco et al. 2012; Laloë et al. 2019).

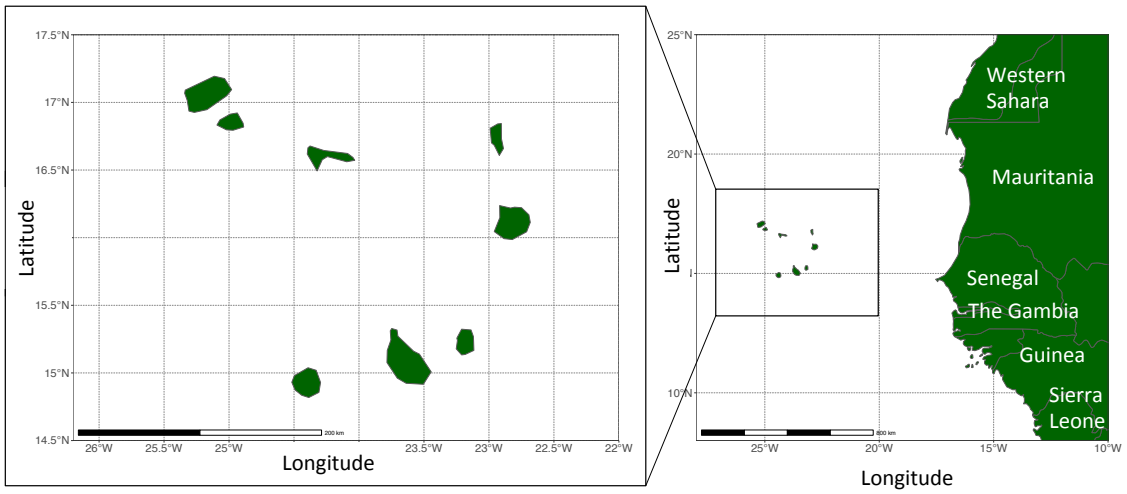

Figure I.5: Cape Verde is an archipelago in the eastern Atlantic, approximately 1000 km from the coast of Senegal

The Cape Verde population of loggerhead sea turtles is comprised of several genetically distinct nesting groups distributed across the archipelago (Stiebens et al. 2013). The feeding strategies in this population include a neritic and oceanic group, with neritic turtles tracked to the coastline of Sierra Leone and oceanic turtles roaming the oceans between Mauritania and Senegal (Hawkes et al. 2006; Eder et al. 2012). Recently, using stable isotope analysis, Cameron et al. (2019) identified a second oceanic foraging strategy, closely linked to oceanic upwelling systems. There is some evidence that these strategies may be linked to imprinting during the passive drift of individuals as hatchlings (Scott et al. 2014B).

A long-term monitoring project across the Cape Verde archipelago was established in 2009, with the aims of i) standardising data-collection methods across all islands, ii) utilising the strength of citizen science to collect samples and iii) obtain long-term genetic and phenotypic data that can contribute to conservation management plans. To date, this project revealed the existence of genetically distinct sub-populations in Cape Verde (Stiebens et al. 2013). As the dataset as grown, it has elucidated the maintenance of diverse feeding strategies within this population (Cameron et al. 2019). Noteworthy,

among the different traits recorded, the presence of the leech parasite *Ozobranchus margoi* has also been systematically monitored since 2009. As this leech is the most likely vector of the sea turtle fibropapilloma virus, this monitoring acts as an early warning system for detection of this lethal condition (Greenblatt et al. 2004; Jones et al. 2016).

6.6 The Key Questions

Global change poses a challenge to all sea turtle species across each stage of their life-cycle. However, existing predictions of population responses to climate change have thus far rarely considered any potential for adaptive or plastic responses to changing environments. Instead, predictions are constrained to taking current thermal responses as a rigid framework. In this thesis, I fill this knowledge gap by revisiting much relied-upon projections of offspring sex ratios in response to climate change. Through a combination of meta-analysis, controlled field experiments and long-term data analysis, I explore whether and to what extent plastic or adaptive mechanisms contribute to thermal response curves at nesting beaches, and how this might impact population viability. I also test for a role of maternally derived sex steroid hormones on sea turtle TSD as a possible buffering mechanism against rising temperatures. Related to change in environmental conditions, I test how nesting females respond to parasite infection, a key facet of hormone mediated immune trade-offs. Particularly, I explore the effect of infection on foraging strategy and reproductive output. Finally, I investigate the role of sex steroid hormones as the proximate mechanism of immune-reproduction trade-offs in sea turtles, and discuss whether a changing environment could disrupt these trade-offs. The overarching aim of this thesis is to determine how sea turtles will respond to climate change, with an ultimate view to influence conservation strategies over the next century.

7. Thesis Outline

This thesis combines chapters on population ecology, physiology and parasitology, and explores how phenotype-environment matching influences the population demography of the seven sea turtle species in the face of global change.

In Chapter 1, I combine the current knowledge of sea turtle TSD response curves in a meta-analysis, to test whether sea turtle populations around the world match their thermal developmental traits to local environmental conditions. I then model the future of this phenotype-environment matching following IPCC (Intergovernmental Panel on Climate Change) prediction under plastic and adaptive scenarios, and revise projections of offspring sex ratio feminisation for the 21st century.

In Chapter 2, I experimentally incubate sea turtle eggs at constant temperatures to i) measure whether there is variation in offspring sex ratios that cannot be attributed to temperature and ii) to assess whether this variation is explained by maternal transfer of testosterone and oestradiol – a key possible mechanism by which sea turtles might express a plastic response to environmental change.

In Chapter 3, I use long-term data on infection rates of a leech parasite of turtles across the Cape Verde archipelago to explore impacts of infection on feeding ecology and reproductive success.

Finally in Chapter 4, having directly considered the role of hormones in TSD and knowing their role in immunity-reproduction trade-offs, I examine turtles' variation in testosterone and oestradiol and how this relates to the environment (sea surface temperature, infection and feeding location). Linking this variation to reproduction in adult females, and locomotion in hatchlings, I test the role of hormones as functional mechanisms of life-history trade offs.

Global Evaluation of the Effects of Climate Change on Sea Turtle Sex Ratios

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Keywords: Climate Change, Temperature-dependent Sex Determination, Sea Turtle, adaptation, plasticity, mathematical modelling

1.1 Abstract

Climate change may impact population dynamics, and forecasting these effects is important for policy and conservation. As temperatures rise, species with temperature-dependent sex determination (TSD), such as sea turtles, risk extinction through extreme sex ratios bias. Because little is known about the adaptive potential of TSD species, predictive modelling must simulate various response scenarios to guide mitigation efforts. In a global meta-analysis, in a space-for-time approach, we combined embryonic thermal response curves from populations of all sea turtle species, and linked them to local environmental conditions at nesting grounds. We found evidence of local adaptation in turtle populations, with 35% of the variation in pivotal temperature (the temperature producing 50:50 male/female offspring) being associated with local air temperature and rainfall during embryonic development. Based on these findings, we predicted offspring sex ratios for thirty populations throughout the 21st century under three scenarios: i) a fixed pivotal temperature of 29 °C, and scenarios that assume the pivotal temperature ii) to be plastic and guided by short term environmental changes or iii) heritable and under natural selection. Under conservative models of IPCC climate warming, even under fully plastic responses, half of the populations studied could produce offspring sex ratios that exceed 90% female by 2100. The rates of these changes would be greatest before 2040. Should temperatures rise more than 2 °C above pre-industrial levels, then we predict over 75% of populations would surpass 90% female offspring. Here, we suggest that even optimistic adaptive potential may be insufficient to prevent local extinction of populations in this taxon.

1.2 Introduction

At the end the current century, conservative estimates of global warming predict temperatures will lie between 0.3 °C and 1.7 °C above pre-industrial averages (Stocker et al. 2013). Without stringent global mitigation, they will most likely exceed 2 °C (Stocker et al. 2013). The consequences of such rapid warming will be profound for ecosystem functioning, species distributions, phenology, and population dynamics (Edwards & Richardson 2004; Perry et al. 2005; Parmesan 2007; Chevin et al. 2010; Walther 2010). The adaptive potential of populations is crucial to determine their responses to these environmental shifts, yet the mechanisms underlying this potential frequently remain unknown (Eizaguirre & Baltazar-Soares 2014). Instead, we must often use proxies representing different scenarios to guide mitigation strategies. Here, mathematical modelling guided by environmental variation can be an important tool for evaluating the effects of possible response mechanisms.

Species that demonstrate temperature-dependent sex determination (TSD) are specifically vulnerable to climate change (Hulin et al. 2009; Mitchell & Janzen 2010; Refsnider & Janzen 2015). For these species, the temperature during a thermosensitive period of embryonic development establishes the sex of embryos (Charnov & Bull 1977). The sex-ratio of a clutch is determined by a logistic thermal response curve defined by two primary characteristics; i) the temperature at which an equal ratio of male and female offspring will develop, known as the pivotal temperature (T_{piv}), and ii) the range of temperatures where both sexes are produced, known as the transitional range of temperatures (TRT) (Mrosovsky & Pieau 1991). If TSD species do not have either evolved mechanisms to adjust their thermal response curves to climate fluctuations or sufficient adaptive potential, global warming will likely result in extreme biases towards a single sex, threatening species persistence (Hawkes et al. 2009; Witt et al. 2010; Laloë et al. 2014). For instance, without phenotypic plasticity or rapid

adaptation to environmental change, all tuatara, *Sphenodon punctatus* and *S. guntheri*, offspring will be male by the year 2085 under current climate prediction models, most probably leading to the demographic collapse of these species (Mitchell et al. 2010).

There is likely a single origin of TSD in vertebrates, approximately 300 million years ago (Janzen & Krenz 2004). This ancient origin suggests that most lineages with TSD have experienced, and survived, previous periods of significant environmental change along their evolutionary history (Mitchell & Janzen 2010; Silber et al. 2011). Accordingly, it is hypothesised that they have evolved mechanisms that enable them to adapt or tolerate temperature variation. Such mechanisms may be genetic, as heritable variation in T_{piv} (up to $h^2 = 0.35$) exists in populations of the painted turtle, *Chrysemys picta* (McGaugh et al. 2011). Additionally, responses to temperature change might be plastic. The population average T_{piv} of the painted turtle also correlates strongly with mean annual air temperatures during the nesting period, suggesting that within individual variation might be responsive to environmental temperatures (Schwanz et al. 2010). Despite evidence of both genetic and plastic responses in model TSD species, they may yet be constrained by the rapid rates of contemporary temperature change (Refsnider & Janzen 2015). No formal evaluation of fixed, heritable or plastic T_{piv} responses to climate change exists, despite such analyses being critical for understanding the mid-term prognosis for endangered taxa such as sea turtles.

In sea turtles, our understanding of the TSD mechanism has been constrained by a lack of non-lethal methods to sex hatchlings due to the highly protected status of many populations. Instead, offspring sex ratios at nesting rookeries are frequently estimated indirectly from thermal response curves that are based on a few *in vitro* incubation studies with small sample sizes (Wyneken & Lolavar 2015) (e.g. Appendix 1, Table A1.1), with the T_{piv} of sea turtles often approximated to lie fixed around 29 °C (Ackerman 1997). Importantly, these studies do not account for other environmental

variables, such as precipitation or humidity, nor do they account for thermal stratification within a natural nest (Lolavar & Wyneken 2015; Wyneken & Lolavar 2015). Because sea turtles are highly philopatric, and nest across a wide range of environmental conditions, populations may have instead locally adapted to match their thermal response (T_{piv} and TRT) to their specific nesting environment. Such patterns of local adaptation have already been illustrated in other TSD species, such as in the American snapping turtle *Chelydra serpentina,* and exist for sea turtles in relation to other traits such as immunity, foraging behaviour, and nesting substrate (Ewert et al. 2005; Stiebens et al. 2013; Liles et al. 2015; Cameron et al. 2019).

In response to climate change, a T_{piv} of 29 °C predicts extreme female biases will occur in some sea turtle populations towards the end of this century (Hawkes et al. 2007; Witt et al. 2010). For instance, over 99% female offspring of loggerhead turtle are expected from some beaches in Cape Verde by 2100 (Laloë et al. 2014; Tanner et al. 2019), and similar extreme biases have been proposed to already exist in green turtle offspring from beaches on the Great Barrier Reef (Jensen et al. 2017). Because existing sex ratio predictions assume no adaptive potential of sea turtle thermal response curves over time, it is possible that these models have systematically underestimated male offspring production. If true, these biases may encourage the use of mitigation strategies, for instance shading of nests to increase the production of male offspring, when unnecessary or even detrimental (Patino-Martinez et al. 2012; Wood et al. 2014).

If there is no differential mortality between the sexes, offspring sex ratios should be reflected in adult sex ratios upon cohort maturity. Sea turtles are polyandrous, and males return to breeding grounds more frequently than females (Lee & Hays 2004; Hays et al. 2010). This mating system results in fewer males than females being required to maintain a viable operational sex ratio (OSR), with 1.4 males for every reproductive female reported in Cyprus (Wright et al. 2012). Populations must, however, still recruit

a critical number of adult males to avoid failure in reproduction and demographic collapse (Hays et al. 2014). Despite the importance of understanding mating systems for conservation management, few direct estimates of OSR in populations exist, owing to the difficulties associated with access to adult male turtles.

Here, using a space-for-time approach, we examined spatial and temporal variation in the T_{piv} and TRT from the published literature, to test for evidence of local adaptation of thermal responses in global sea turtle populations. We then explored whether different possible adaptive scenarios might result in different rates of population feminisation as global temperatures rise. To do this, we compiled published population-specific thermal response curves from 37 nesting populations of sea turtle including all seven species. We predicted that in warm regions, local adaptation would result in a higher T_{piv} to increase the likelihood that male offspring are produced. Since rainfall has a cooling effect, we expected high levels of precipitation to be associated with a reduced T_{piv}. Where environmental conditions are highly variable among years, we hypothesised turtles have evolved a wide TRT to encompass a larger thermal niche, and prevent extreme within-year biases towards production of one sex. We finally incorporated these hallmarks of adaptation into projection models to predict sex ratios over the 21st century under three theoretical scenarios: i) a fixed T_{piv} of 29 °C as commonly modelled (Hawkes et al. 2007; Laloë et al. 2014), ii) a plastic T_{piv} that tracks short-term environmental conditions and iii) a heritable T_{piv} that allows for some environmental tracking but at a more constrained rate than a plastic response. We applied these scenarios to two possible IPCC projections; one based on stringent mitigation of carbon emissions, where temperatures are unlikely to exceed 1.5 °C (RCP 2.6) and one based on intermediate mitigation measures (RCP 6.0), under which global temperatures increase by 1.4 to 3.1 °C.

1.3 Results

1.3.1 Thermal response curves and latitude

Through two independent literature searches by the authors using online academic search engines, we collated studies that reported the direct measurements of T_{piv} and TRT of sea turtle populations from experimental studies that sacrificed hatchlings to determine sex ratio. As experimental design differed, we excluded those studies that i) manipulated temperature during incubation (n = 2); ii) involved the application of hormones to eggs (n = 2); or iii) manipulated multiple environmental conditions (n = 3). After these criteria were applied, our final dataset (Appendix 1, Table A1.2, n = $37_{populations}$) spanned 40 years (between 1978 and 2017) and 70 degrees of latitude, from -29.99 N to 39.38 N. The data collected follow the natural distribution pattern of four out of seven species: loggerhead *Caretta caretta*, green *Chelonia mydas*, olive ridley *Lepidochelys olivacea* and hawksbill *Eretmochelys imbricata* turtles (Appendix 1, Figure A1.1). Available data for the remaining three extant sea turtle species, the leatherback *Dermochelys coriacea*, Kemp's ridley *Lepidochelys, kempii* and flatback *Natator depressus* turtles, did not cover the entire extent of their range, but were still included in analyses.

For 31 of the 37 studies, we extracted information on the sex ratios produced under different temperatures, and re-calculated the T_{piv} and TRT using the Hill equation (which accounts for logarithmic scaling in the shape of the TRT (Fuentes et al. 2017)) within the R package *embryogrowth* (Girondot 1999). This allowed us to calculate the TRT where not originally reported, and to standardise it as the range of temperatures between which 5 and 95% female offspring are produced. There was no correlation between T_{piv} and TRT ($F_{1,27} = 0.203$, $p = 0.656$). We found a species effect for T_{piv} (Appendix 1, Figure A1.2: $F_{6,30} = 8.246$, $p < 0.001$), with a post-hoc Tukey test showing

the T_{piv} values of olive ridley turtles were significantly higher than other species. There were no species differences in TRT ($F_{6,22} = 2.331$, $p = 0.068$).

As expected from our local adaptation hypothesis, absolute latitude significantly predicted variation in T_{piv} and TRT between populations. Of the variation in $T_{piv,}$ 11.6% was explained by latitude, with values decreasing from 30.02 °C near the equator by an estimated 0.27 °C for every ten degrees of increasing latitude ($F_{1, 35} = 5.703$, $p = 0.022$, Figure 1.1A). In contrast, TRT positively correlated with latitude ($F_{1,27} = 5.377$, $p = 0.028$, Figure 1.1B), possibly due to the greater thermal variation that exists among years in more temperate regions. These results are consistent with our hypothesis that local adaptation modulates the TSD traits of sea turtles.

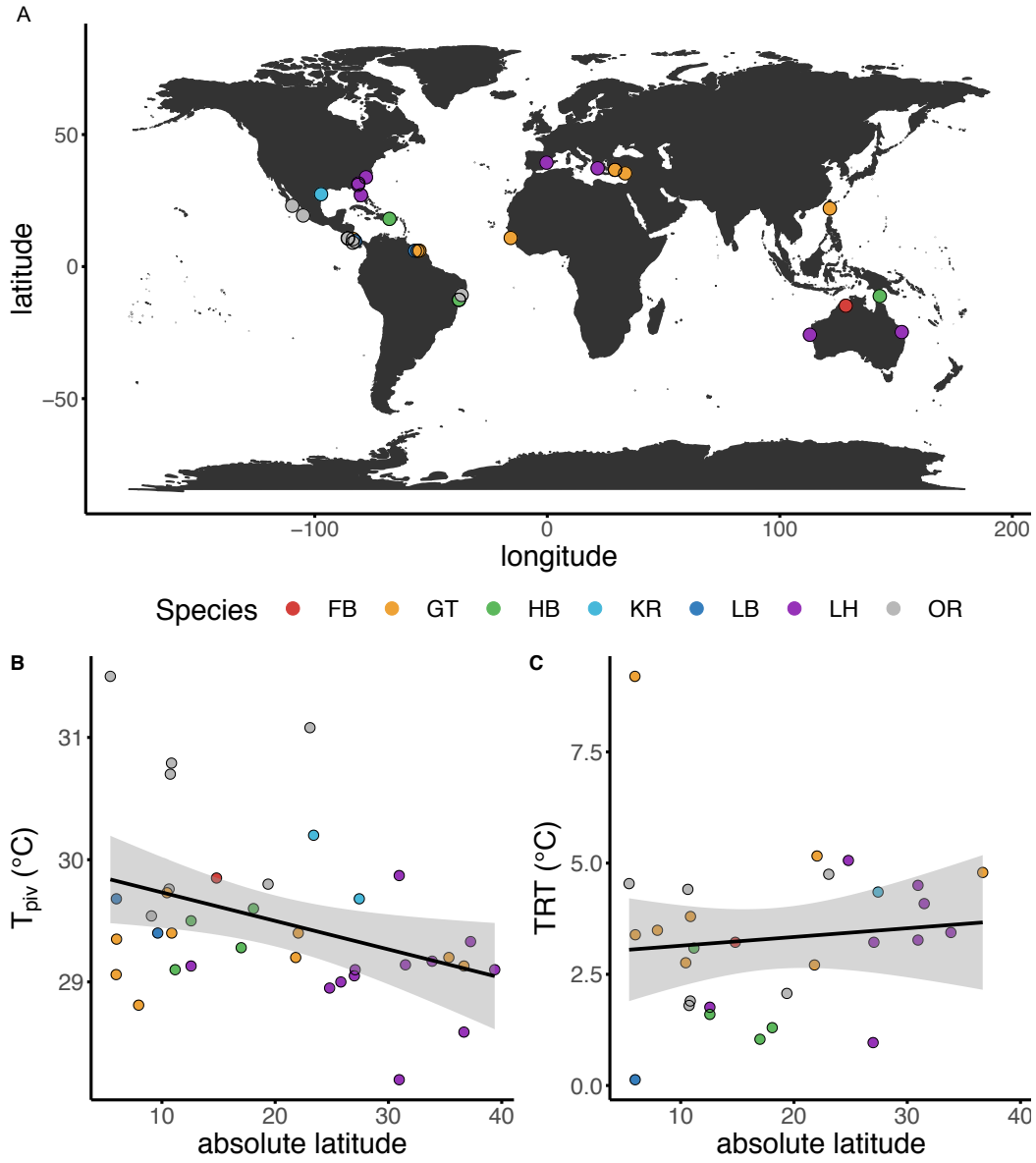

Figure 1.1: A) Map of rookeries used within analysis FB = flatback, GT = green, hb = hawksbill, KR = Kemp's ridley, LB = leatherback, LH = loggerhead, OR = Olive ridley B) Scatterplot showing a significant negative relationship between the absolute latitude of a rookery, and its population pivotal temperature (T_{piv}, $F_{1, 35}$ = 5.703, p = 0.022), and C) Scatterplot showing a positive relationship between absolute latitude and population transitional range of temperature (TRT, $F_{1,27}$ = 5.377, p = 0.028).

1.3.2 Environmental conditions and thermal response curves

To understand the drivers of latitudinal patterns of TSD traits, we obtained environmental data that detailed the average monthly temperature (NOAA National Climatic Data Centre, Terrestrial Air Temperature V.4.01) and total monthly rainfall (NOAA Climate Prediction Centre) recorded at the specific rookeries. Specifically, we focused on the environmental values for the thermosensitive month of the clutches from which thermal response curves had been derived. Using linear models, we tested how mean monthly temperature and total monthly rainfall, along with their interaction, predicted both T_{piv} and TRT of the studied populations (Table 1.1). The interaction between temperature and rainfall during the thermosensitive month explained 34.5% of overall variation in population T_{piv} ($F_{1,27} = 12.142$, $p = 0.002$, Figure 1.2). We found a 0.26 °C increase in T_{piv} per 1 °C increase in air temperature, but this relationship was moderated by rainfall. For rookeries with air temperatures below 25 °C, rainfall elevated the T_{piv}, whereas for rookeries with air temperatures above 26 °C, rainfall reduced T_{piv}. Based on the slopes and intercept defined by our model, the population-specific T_{piv} can therefore be estimated as:

$$T_{piv(i)} = 0.257t_i + 0.0389P_i - 0.0015t_iP_i + 22.787 \quad \text{... } \textit{Eq. 1}$$

where t_i is the monthly mean air temperature during the thermosensitive period, and P_i is the total monthly precipitation during this same period. There was no relationship between temperature or rainfall during the clutches' thermosensitive month and TRT (Table 1.1).

Table 1.1: Relationships between environmental conditions during the thermosensitive month at a rookery, and the T_{piv} and TRT of study clutches. Significant relationships are shown in bold.

	d.f.	F	p
T_{piv}			
Air Temp	**1**	**5.800**	**0.023**
Precipitation	1	0.857	0.363
Air Temp*Precipitation	**1**	**12.142**	**0.002**
TRT			
Air Temp	1	0.376	0.546
Precipitation	1	1.271	0.272

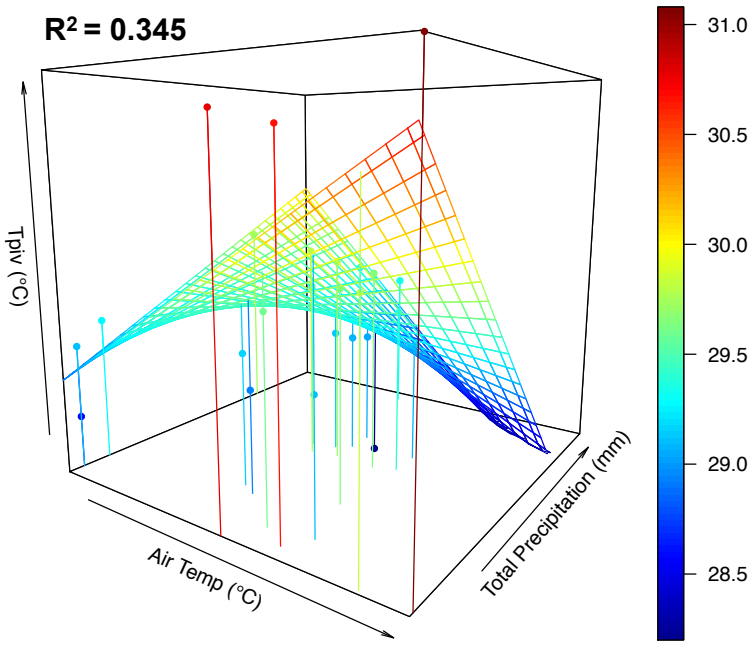

Figure 1.2: 3D scatterplot showing a significant interaction between the effects of air temperature and total precipitation on pivotal temperature (T_{piv}, $F_{1,27}$ = 12.142, p = 0.002)

If temperature and rainfall conditions are particularly variable among years, we hypothesised that a wide TRT should evolve to allow the thermal response curve to encompass a greater environmental niche. Such an evolved trait could avoid biased offspring sex ratios during years of extreme weather conditions. We collated the average air temperature and total precipitation levels of the study clutch thermosensitive month between 1979 and 2014, and calculated the standard deviation of these variables within this time period. A linear model showed that populations exposed to greater variability in air temperature ($F_{1,24} = 5.293$, $p = 0.030$) and rainfall ($F_{1,24} = 5.030$, $p = 0.034$) had a wider TRT than populations from regions with highly predictable climates (Table 1.2). Neither variability in air temperature nor precipitation predicted T_{piv} (Table 1.2: temperature: $F_{1,32} = 0.939$, $p = 0.339$; precipitation: $F_{1,32} = 0.682$, $p = 0.415$). Together, our findings therefore suggest TRT and T_{Piv} could have evolved independently to different aspects of local climate.

Table 1.2: Relationships between both T_{piv} and TRT and the standard deviation of average temperatures and total rainfall within the thermosensitive month of study clutches over between 1979 and 2014. Significant relationships in bold.

	d.f.	F	p
Tpiv			
Air Temp	1,32	0.939	0.339
Precipitation	1,32	0.682	0.415
TRT			
Air Temp	**1,24**	**5.293**	**0.030**
Precipitation	**1,24**	**5.032**	**0.034**

1.3.3 Offspring sex ratios under climate change scenarios

As predicted by local adaptation, we showed that air temperature and precipitation at nesting sites explained significant levels of variation in the key parameters of the response curve relating incubation temperature to sex ratio across global sea turtle populations. This could be explained by some element of plasticity of these parameters to short-term environmental variation, or alternatively could be the consequence of selection on heritable traits. We thus considered how evolved traits might affect sex ratios differently under climate change, by modelling three theoretical scenarios.

First, despite finding evidence for local adaptation of T_{piv}, and the high likelihood that sea turtles might be able to further evolve over time, scenario 1 modelled offspring sex ratios under a fixed T_{piv} across time, with the assumption that this trait would not evolve or exhibit plasticity. We model this projection to test the possible variation emerging from a study solely focusing on modelling sex ratios without experimentally determining the T_{piv}. We chose to use a fixed T_{piv} value of 29 °C, in line with previous studies focusing on single populations (e.g. Laloë et al. 2014). An alternative model with fixed rookery-specific T_{piv} is shown in the supplementary material (Appendix 1, Figure A1.3). We believe this alternative model is less intuitive, as it would assume T_{piv} evolved to be rookery specific but would not allow for any future evolution/change.

Secondly, Scenario 2 assumed a rookery specific, plastic T_{piv} which responded to short-term environmental conditions. Rookery T_{piv}s were recalculated yearly, using equation 1 and predicted yearly local air temperatures and precipitation averages. To ensure the T_{piv} recorded in the original study was included, we fit all predicted T_{piv}s in relation to the original T_{piv}. Finally, to test how the slope and intercept parameters of equation 1 influence the qualitative outcome, we also tested the 95% confidence intervals of this model. Such a plastic response could be, for instance, the result of differential maternal

investments in sex steroid hormones influencing the pivotal temperature of clutches, which has been described in the painted turtle (Bowden et al. 2000; Carter et al. 2017).

Finally, scenario 3 assumed that the environment – thermal response curve match is constrained by trait heritability. Here we define heritability as the theoretical positive slope between maternal T_{piv} and offspring T_{piv}. We fixed this slope at 0.351, as reported in the painted turtle under field conditions (McGaugh et al. 2011), resulting in a T_{piv} shift of 35.1% of the yearly change predicted by the plastic response in Scenario 2, since reproduction (i.e. new recombination of alleles) happens yearly.

Under these three scenarios, we modelled change in offspring sex ratios during the thermosensitive month of study clutches across the 21st century, in response to two different projections reported in the Fifth IPCC Assessment Report (Stocker et al. 2013). We first forecast offspring sex ratios under stringent mitigation of carbon emissions (Representative Concentration Pathway (RCP) 2.6) that would likely prevent global warming exceeding 2 °C above pre-industrial levels. Secondly, we selected a more severe scenario for projections, RCP 6.0, where warming will be between 1.4°C and 3.1 °C by the year 2100.

To calculate offspring sex ratios, we first quantified the relationship between air and nest temperatures. Sand temperature strongly correlates with air temperature (eg. r^2 between 0.73 and 0.84 in Cape Verde (Laloë et al. 2014)). We show that this relationship also predicts nest temperatures by using mean daily nest temperatures from 28 temperature loggers placed at the centre of incubating clutches in Cape Verde during 2017 (Appendix 1, Figure A1.4, $F_{1,69} = 92.833$, $p < 0.001$). To create a heuristic universal equation that can be used globally to predict nest temperature from air temperature, we combined data on the relationship between sand and air temperatures from our nest temperature data and previously studied locations (Esteban et al. 2016). Since intensity and temporal occurrence of precipitation events are not reflected by the

total monthly precipitation value used here, and that it did not rain during our Cape Verde study, we did not include rainfall in our modelled nest temperatures. The universal relationship between sand and nest temperatures was therefore defined as:

$$\tau_i = 0.814t_i + 7.872 \quad \text{... Eq. 2}$$

where τ_i is the nest temperature, and t_i is the monthly mean air temperature (Appendix 1, Figure A1.5). We then used the logistic equation described by Girondot (1999) to estimate sex ratio:

$$S_i = \left| \frac{R_i}{2\log\left(\frac{0.05}{0.95}\right)} \right| \quad \text{... Eq. 3}$$

$$Sr_i = 1 - \frac{1}{1 + e^{\frac{1}{S_i} * (K_i - \tau_i)}} \quad \text{... Eq. 4}$$

where S_i is the shape of the transition from masculinising to feminising temperatures, R_i is the TRT, Sr_i is the sex ratio, K_i is the T_{piv} and τ_i is the nest temperature (Girondot 1999).

We found that a fixed T_{piv} of 29 °C predicts that 71% of the populations included within this study would produce more than 90% female offspring by the year 2100 under conservative climate change (Appendix 1, Figure A1.6), rising to 79% of the populations if temperature increases should exceed 2 °C above pre-industrial levels (Figure 1.3). Hawksbill, Kemp's ridley and flatback turtles are most likely to be at risk, with all populations included here predicted to produce in excess of 99% female offspring by the year 2100 under both RCP projections. Interestingly, under a fixed T_{piv} of 29 °C, populations of these species were already estimated to be producing more than

95% female hatchlings in the year 2000, and thus little change actually occurs across the 21st century. Overall, it is likely that neither plastic response nor heritable adaptation will be able to compensate sufficiently to facilitate male offspring production above 5% in these populations, even under conservative climate change predictions. Should global warming surpass 2 °C, our models predict that fewer than 0.08% of hawksbill turtle offspring would develop as male in 2100, and only 0.02% male offspring for the single flatback turtle population modelled here.

In loggerhead, green and olive Ridley sea turtles, both adaptive scenarios forecasted more male offspring than estimated from the traditional approach assuming a fixed, T_{piv} of 29 °C, because a shift in T_{piv} in response to changes in environmental temperatures maintains male production. As expected from the phenotype-environment match of a plastic response, the shift in T_{piv} over the 21st century was significantly higher than that of a heritable response (t-test, RCP 2.6: $df = 33.553$, $t = -5.064$, $p < 0.001$, RCP 6.0: $df = 33.553$, $t = -4.558$, $p < 0.001$, Fig A1.7, Table A1.3). Despite changes of up to 0.87 °C in T_{piv} across the 21st century under projections from RCP 6.0, by 2100 the two adaptive scenarios predict very little difference in overall offspring sex ratios, with sex ratios becoming increasingly female-biased under both climate projections.

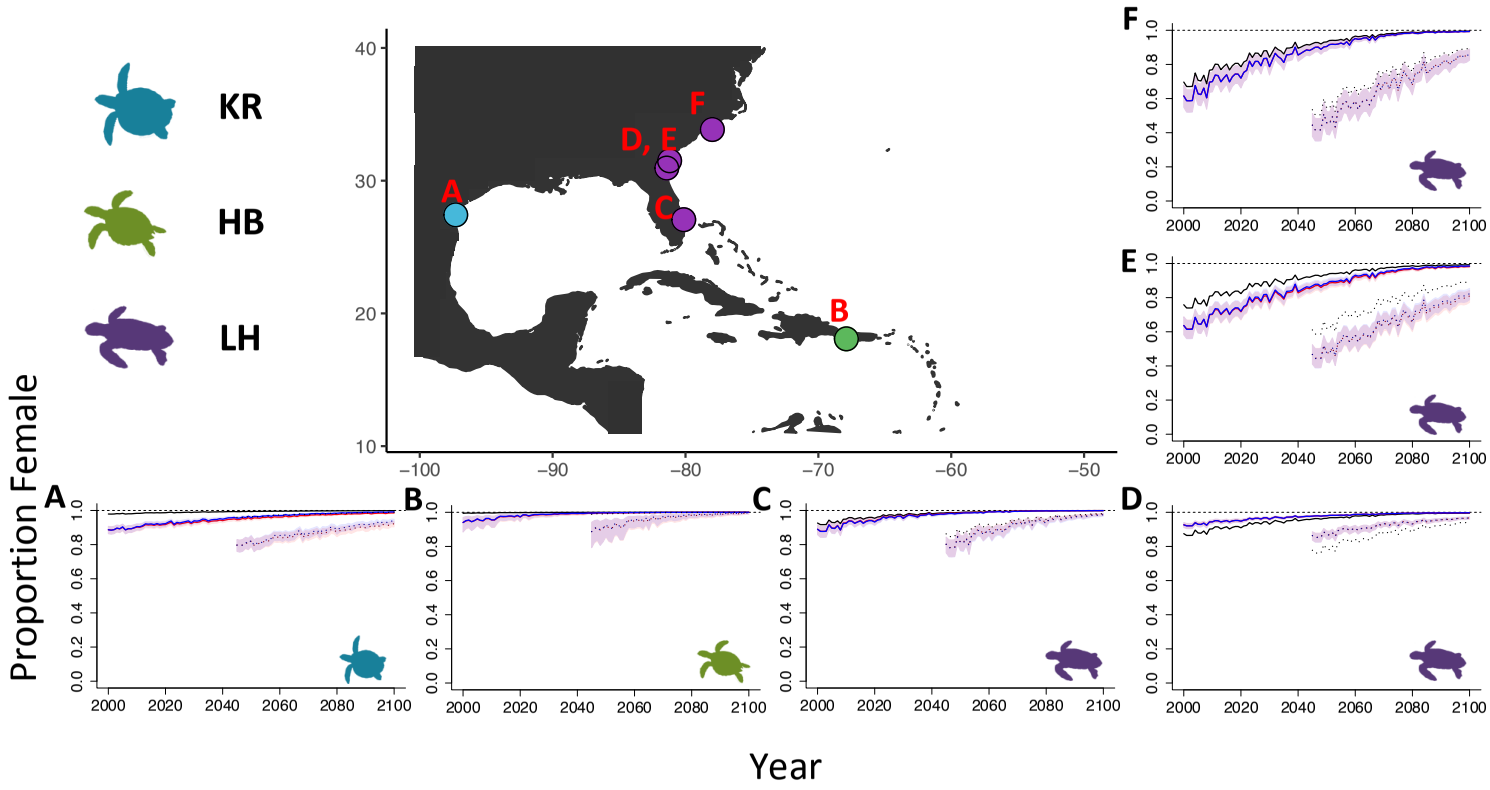

Figure 1.3(i): Time series showing proportion of female hatchlings produced at rookeries in North America and the Caribbean under RCP 6.0, for Kemp's ridley (KR), hawksbill (HB) and loggerhead (LH) sea turtles in A) Texas (LeBlanc et al. 2012) B) Puerto Rico (Mrosovsky et al. 2009), C) Florida (Mrosovsky 1988), D & E) Georgia (LeBlanc et al. 2012; Mrosovsky 1988) and F) North Carolina (Mrosovsky 1988). Black show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

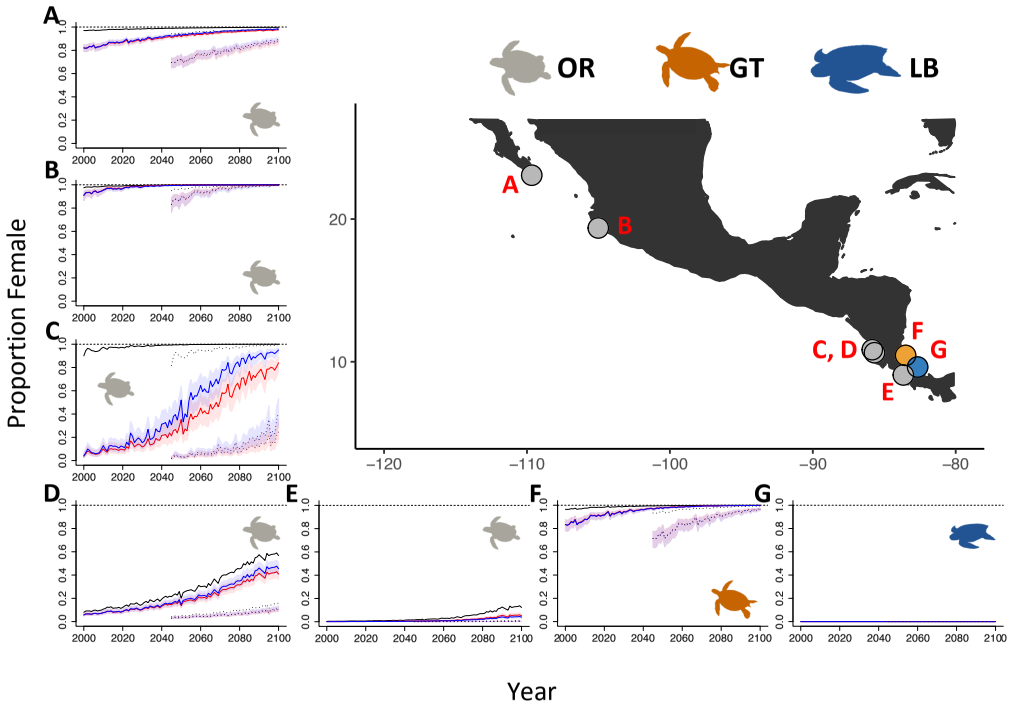

Figure 1.3(ii): Time series showing proportion of female hatchlings produced in Central America under RCP 6.0 for olive ridley (OR), green (GR) and leatherback (LB) in A) North Mexico (Sandoval Espinoza 2012) B) Mexico (Batiz 1986), C, D & E) Costa Rica(Pacific) (Wibbels et al. 1998; McCoy et al. 1983; Brenes Arias et al. 2009) F & G) Costa Rica (Atlantic) (Spotila et al. 1987; Binckley et al. 1998). Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

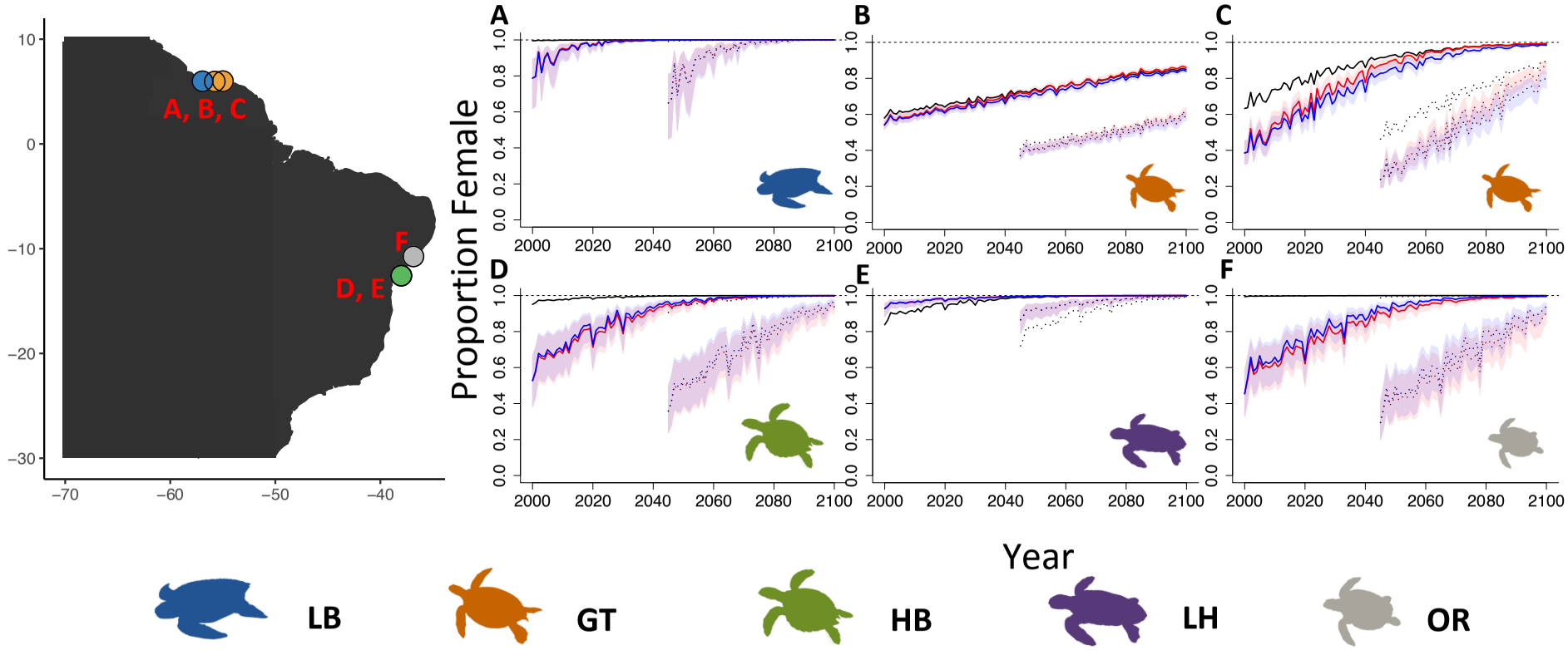

Figure 1.3(iii): Time series showing proportion of female hatchlings produced in South America under RCP 6.0 for leatherback (LB), green (GR), hawksbill (HB), loggerhead (LH) and olive ridley (OR) sea turtles in A) French Guiana (Rimblot et al. 1985) (R2) B & C) Suriname (Mrosovsky et al. 1984; Godfrey & Mrosovsky 2006), D, E & F) Brazil (Godfrey et al. 1999; Marcovaldi et al. 1997; Castheloge et al. 2018). Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

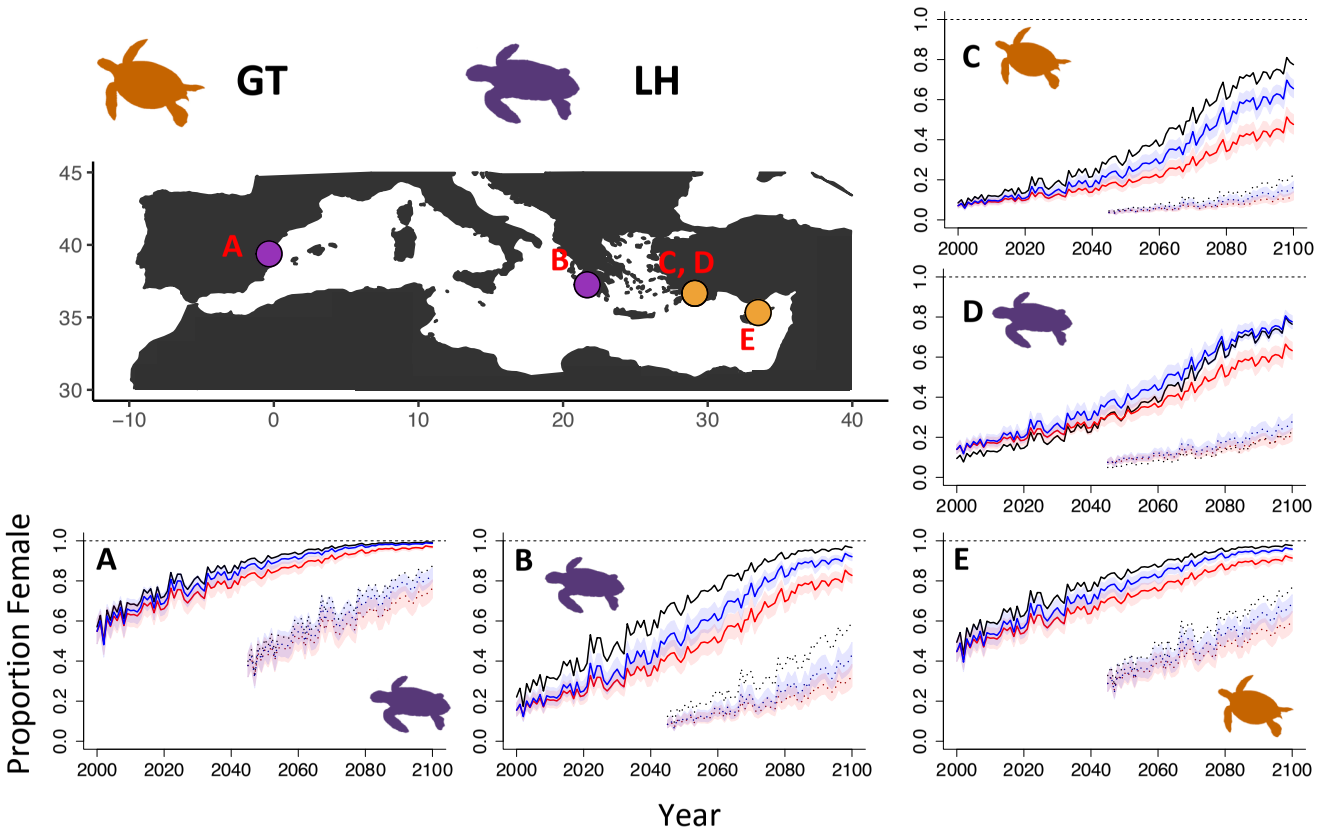

Figure 1.3(iv): Time series showing proportion of female hatchlings produced in the Mediterranean under RCP 6.0 for green (GR) and loggerhead (LH) in A) Spain (Segurado 2016) B) Greece (Mrosovsky et al. 2002) C & D) Turkey (Kaska et al. 1998) and E) Cyprus (Broderick et al. 2000). Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

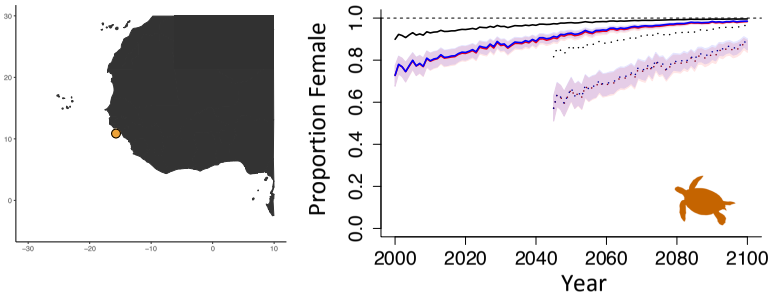

Figure 1.3(v): Time series showing proportion of female green turtle hatchlings produced in Guinea Bisseau (Patrício et al. 2017) under RCP 6.0. Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

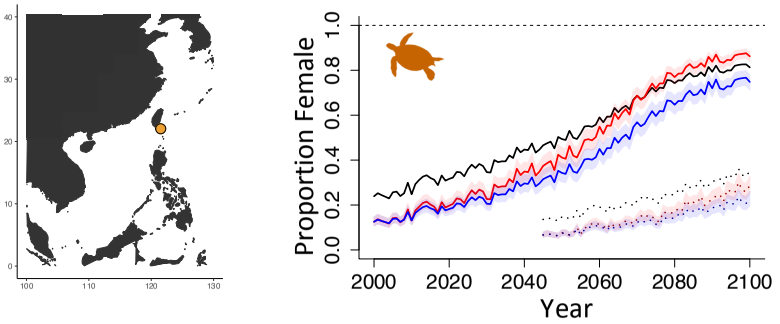

Figure 1.3(vi): Time series showing proportion of female green turtle hatchlings produced in Taiwan (King et al. 2013) under RCP 6.0. Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

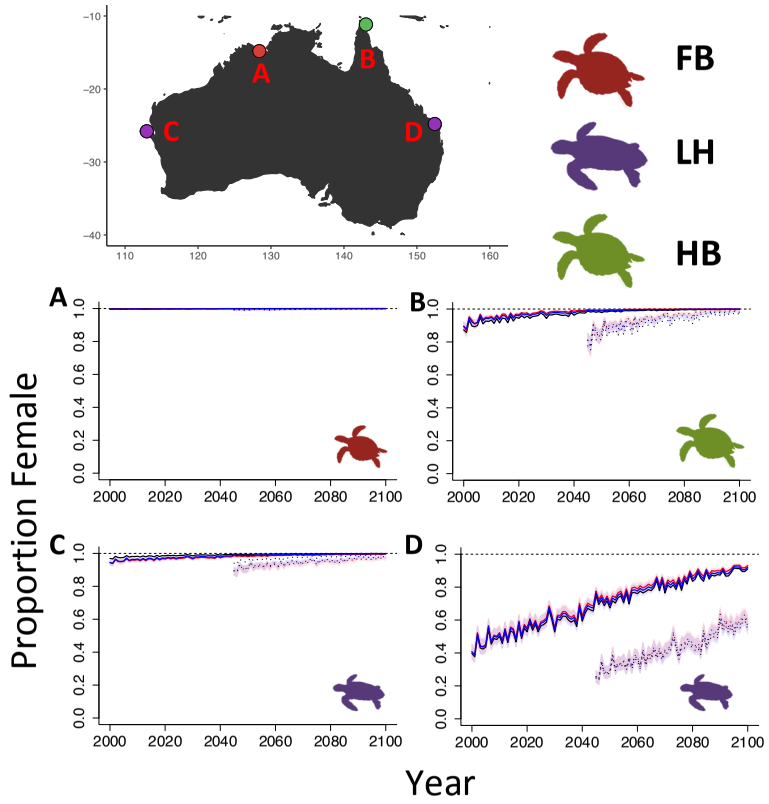

Figure 1.3(vii): Time series showing proportion of female hatchlings produced in Australia under RCP 6.0 for flatback (FB), loggerhead (LH) and hawksbill (HB) sea turtles in A) north Western Australia (Stubbs et al. 2014), B) northern Queensland (Dobbs et al. 2010) C) Western Australia (Woolgar et al. 2013) and D) Queensland (Georges et al. 1994). Black lines show the projected proportion of female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to operational sex ratios (OSR) for the three scenarios.

Between 2000 and 2010, we predict that loggerhead, green and olive ridley turtles are producing 72.54 ± 23.63 (SD)%, 66.44 ± 26.31(SD)% and 66.58 ± 47.89 (SD)% female offspring respectively. Even if we assume that stringent mitigation of carbon emissions will constrain global temperature rise to between 0.3 and 1.7 °C, 50% of sea turtle populations are forecasted to produce over 90% female offspring by 2100 under either adaptive response scenario. This includes 44% of loggerhead populations, 17% of green populations, and 50% of olive ridley and leatherback sea turtle populations in our database. In RCP 2.6, changes in hatchling sex ratios would occur between 2000 and 2040, before global temperature rise begins to plateau. Shifts in sex ratio will likely be greatest for green turtles - a heritable scenario predicts that on average 21.1 ± 10.9(SD)% more female offspring would be produced in comparison to the year 2000, while a plastic response forecasts a similar increase of 21.9 ± 12.1(SD)%.

If global temperatures increase by more than 2 °C above pre-industrial records, the proportion of female offspring is unlikely to plateau as seen under conservative warming, but instead will continue to rise towards the end of the century. Under these conditions, neither environmental plasticity nor genetic heritability could prevent between 75% (plastic scenario) and 82% (genetic heritability scenario) of populations producing over 90% female offspring during this focal month of their nesting season. For example, under RCP 6.0, we predict the average sex ratio of all loggerhead populations included within this study will be between 96.09 ± 6.37(SD)% (plastic) and 97.36 ± 3.89(SD)% (genetic heritability).

Finally, we predicted how these offspring sex ratios would translate into the operational sex ratio (OSR – the ratio of sexually mature males to females) of cohorts upon maturity, assuming no differential mortality between the sexes. Following the simplifying assumption that males breed twice as frequently as females (Hays et al.

2014), but see Table 1.3 for alternative published OSRs, we used the calculation from Laloë et al. (2014) to determine sex ratios with a 45 year lag to maturity as an example:

$$OSR(t_{i+45}) = 100 \times \frac{Sr(t_i)_{females}}{Sr(t_i)_{females} + 2 \times Sr(t_i)_{males}} \quad \dots Eq\ 5$$

Table 1.3: Examples of sea turtle operational sex ratios worldwide

Study	Location	Species	OSR (%M)	Method
(Graeme C. Hays et al. 2010)	Zakynthos, Greece	Loggerhead	47%	Tracking and photo-id
(Wright et al. 2012)	Cyprus	Green	58.3%	Paternity analysis
(Lasala et al. 2013)	Georgia, USA	Loggerhead	72.6%	Paternity analysis
(Stewart & Dutton 2014)	US Virgin Islands	Leatherback	50.5%	Paternity analysis
(Schofield et al. 2017)	Zakynthos, Greece	Loggerhead	50%	Drone surveys

Based on these assumptions, the feminisation of offspring is reflected in the OSR as these cohorts achieve maturity, and will likely not be prevented by differences in mating periodicity. Indeed, by the end of the century under RCP 2.6, our models suggest that cohorts of Kemp's ridley, flatback and hawksbill turtles being recruited to the OSR will be over 90% female, regardless of the mechanisms underlying possible adaptive responses. Going further, under RCP 6.0 projections this figure increases to over 95% for hawksbill turtle populations, and 99% for the flatback turtle. Assuming the thermal response curve reacts to environmental change, in the year 2100 other species may fare better. For example, the models suggest that under RCP 6.0 loggerhead turtle cohorts recruited to the OSR will be 72.94 ± 29.09(SD)% female if T_{piv} responses are plastic, and 75.05 ± 26.82(SD)% female if heritable. Yet, based on the lag-time and the positive trajectory of offspring sex ratios, this OSR feminisation will continue through into the next century.

1.4 Discussion

While sea turtles spend most of their life in the ocean, their highly accurate philopatric behaviour, reducing gene flow among rookeries and populations, has the potential to result in local adaptation (Weber et al. 2012; Stiebens et al. 2013). Here, we showed that T_{piv} and TRT appear to be locally matched to the temperature and precipitation conditions of turtles' nesting regions. Using these findings, we updated sex ratio projection models in response to contemporary climate change around the world.

There was some variation in the T_{piv} among species, with those reported for olive ridley and Kemp's ridley turtles being higher than other species, but no species differences in TRT. Instead, T_{piv}s and TRTs are likely the results of adaptation to different facets of the local environment. Interestingly, as much as 35% of variation in the T_{piv} among populations globally can be explained by an interaction between local air temperatures and precipitation during the thermosensitive month of incubation. Higher air temperatures correlate with higher nest temperatures and higher T_{piv}. Simultaneously, high levels of precipitation reduce sand temperatures through evaporative cooling, and T_{piv} is consequently lower in response. This result complements findings of local adaptation and plasticity in the T_{piv} of other laboratory-TSD species, such as the positive correlation that exists between the population level T_{piv} and annual nesting temperatures in painted turtles (Schwanz et al. 2010). While TRT and environmental conditions within years at rookeries included in our study did not correlate, we did find that TRT is widest in regions where among-year variation in temperature and precipitation at a nesting site is high. This presumably maximises the likelihood that environmental conditions within a given year will fall within the boundaries of the TRT, resulting in both sexes being produced. Compiling known thermal response curves for sea turtle populations globally allowed us to empirically quantify levels of environment-phenotype matching without the need for further, lethal, experimentation.

Local adaptation for other traits has also been reported in sea turtles. Island-specific genetic structure of immune genes and related differences in foraging strategy has been linked to philopatric behaviour within the loggerhead sea turtle population of Cape Verde (Stiebens et al. 2013; Cameron et al. 2019). The high nesting site fidelity of sea turtles suggests that local adaptation may be extraordinarily fine-scale in these species (Weber et al. 2012, Cameron et al. 2019). Artificial incubation of eggs from beaches on Ascension Island found that hatchlings from hot, black sand beaches had greater growth rates at high temperatures than those originating from nests on neighbouring cooler, white sand beaches (Weber et al. 2012). This pattern was attributed to local adaptation of thermal reaction norms at a resolution of only several kilometres (Weber et al. 2012). Such studies demonstrate the potential strength of selection associated with philopatric behaviour, as suboptimal conditions would result in strong selection against individuals with poor fitness.

Despite local adaptation of other ecologically relevant traits in sea turtle populations, variation in the TSD thermal response curve across regions has rarely been considered (Weber et al. 2012; Stiebens et al. 2013; Cameron et al. 2019). A comparison of the T_{piv} in loggerhead turtles nesting along the eastern coast of the USA found no difference between the T_{piv} of turtles nesting in Florida and those in North Carolina (Mrosovsky 1988). Mrosovsky's (1988) study, however, spanned only seven degrees of latitude, while we report results that span sixty degrees for loggerhead turtles specifically, and seventy degrees overall. Our global perspective suggests that differences in thermal response curves across latitudinal and environmental gradients exist, which have previously gone unobserved at smaller geographic scales. We anticipate that the variation reported here might be used to define the thermal response curve for populations where direct estimates are unavailable. This could increase the scope and

potential accuracy of sea turtle sex ratio estimates and modelling without requiring studies that sacrifice offspring, thus enabling appropriate conservation management.

Adaptive potential exists in the T_{piv} and TRT across large geographical scales. For this pattern to emerge, selection must have occurred to match these traits to local environmental conditions. Our mathematical models that project future sex ratios demonstrate, however, that even if T_{piv} were to plastically respond to local thermal variation on a yearly basis, this would likely be insufficient to prevent almost total feminisation in most populations if global temperatures increase by more than 2 °C. This is because of an apparent constraint on T_{piv} plasticity indicated by our model whereby for every degree of increase in air temperature, T_{piv} is unlikely to increase by more than 0.26 °C. While the scope of this study is unable to identify the biological mechanism constraining T_{piv} plasticity, this could possibly be due to a limitation in maternal hormone transfer – known to cause plasticity in T_{piv} in painted turtles (Bowden et al. 2000). Such a response-lag slows down the potential for T_{piv} to match concomitant increases in air temperature, as the environment deviates from the optimum for contemporary thermal response curves (Gienapp et al. 2008; Chevin et al. 2010).

In this study, we have predicted the offspring sex ratios of populations during one month of their nesting season. As environments will vary across a nesting period, thermal refugia may exist for the production of males during cooler nesting months, should they occur. The relative change in sex ratio will, however, be mirrored across all months, and so it is reasonable to conclude that overall male production will be reduced in comparison to current sex ratios. Alternative responses to prevent total feminisation would require a change in phenology, distribution or nesting behaviour (Hawkes et al. 2009; Witt et al. 2010). The median nesting date of loggerhead sea turtles in Florida was ten days earlier by 2003 than in 1989, and this correlated negatively with sea surface temperatures (Weishampel et al. 2004). Yet, despite evidence of phenological shifts,

models suggest these will likely be insufficient to alleviate the effects of temperature increases during the thermosensitive period, due to the rate of current climate change (Telemeco et al. 2013).

In the future, new geographical areas may become suitable for nesting, provided coastal development is controlled (Pike 2013). Despite their philopatric behaviour, sea turtles have the dispersal capacity to colonise new habitats, but large population sizes must exist for exploration behaviours to emerge (Mills & Allendorf 1996). Fine scale nesting behaviour such as nest location, depth and substrate choice could also alter the thermal incubation environment of clutches (Refsnider et al. 2013; Reneker & Kamel 2016). Indeed, in Guinea Bissau 60% more males are produced when nests are laid in forested areas, balancing offspring sex-ratios overall (Patrício et al. 2017). However, nest site selection appears to be conserved within individuals, which might constrain nesting females' potential to adjust behaviour in response to environmental conditions (Reneker & Kamel 2016). Altogether, the limited evident capacity for sea turtle behavioural responses to temperature change strengthens the general assertion that climate change poses a real threat to sea turtle sex ratios (Hawkes et al. 2009; M. J. Witt et al. 2010; Reneker & Kamel 2016).

The heavily biased offspring sex ratios could eventually be reflected in OSRs upon cohort maturity. By the end of the century, our models suggest that adult males will still recruit to the OSR in many populations, but this proportion will continue to decrease after 2100. We speculatively assumed that turtles take an average of 45 years to reach sexual maturity (Laloë et al. 2014). Reducing this age will only reduce the time in which feminisation of the OSR will occur and not the long-term impact of feminisation. Importantly, this is likely to be in a non-linear manner, as the overlap of older cohorts with new recruits will likely increase if age at maturity decreases, effectively slowing the rate at which feminisation occurs. The slow maturation rate of these species

suggests that adult males will probably still be recruiting to the reproductive population at the end of this century, but eventually all OSRs tending to become heavily female biased. It is well known that the effectiveness of differences in mating periodicity at balancing OSRs is weakened when sex ratios become extremely biased, since there is a ceiling effect on individual male reproductive capacity (Hays et al. 2014). For example, while a hatchling sex ratio of 97.5% female offspring will produce a slightly less biased OSR of 95.1% females, at these levels the likelihood of male-female encounters will be reduced (Hays et al. 2014). To date, there has been no quantitative estimate of how many males are required to maintain sea turtle populations, and this point will be key for determining how TSD may affect future dynamics and informing management plans (Laloë et al. 2014; Hays et al. 2014). However, even if sufficient males exist for fertilisation to occur, a reduction in numbers might increase the risks of inbreeding, and reduce population heterozygosity (Willi et al. 2006; Willi & Hoffmann 2009)

Until now, there has been no quantification of local adaptation in the TSD mechanism of sea turtles, or consideration as to how this might influence the effects of climate change. Here, we modelled the limits of local adaptation (either of plastic or genetic origin) of the T_{piv} and TRT of sea turtle populations in response to local environmental conditions at nesting grounds. However, the rapid rate of climate change appears to make any physiological adaptations insufficient to mitigate effects on population dynamics of these threatened species. Our results confirm earlier predictions that climate change risks extreme feminisation of sea turtle populations, but improve the accuracy of the projections and thus will improve the information available to conservationists implementing management for species recovery. In the past, the use of a fixed T_{piv} has probably resulted in inaccurate estimates, which may have negative consequences for population management strategies. Perhaps more importantly, however, we show that if any form of adaptive response exists, either plastic or genetic,

it will probably be insufficient to prevent feminisation. Finally, we show that the next 20 to 30 years will be crucial for avoiding extreme biases in sex ratio, with our study providing a global perspective on the effect of climate change on sea turtle demographics. We conclude that in 2100, total global production of male sea turtles is likely to be exceedingly low.

1.5 Methods

1.5.1 Thermal Response Curve Data Collection

Two independent literature searches were completed (E.L and P.R.T) to collate peer-reviewed studies that reported findings of both/either T_{piv} and TRT within sea turtle populations. The searches were conducted using Google Scholar (www.scholar.google.com), SCOPUS (www.scopus.com) and Web-of-Science (www.webofknowledge.com), and included search terms such as "sea turtle", "pivotal temperature", "sex ratio" and "temperature-dependent sex determination". Any relevant papers cited by these studies were also incorporated, along with several PhD theses. While it is possible that this approach was not exhaustive, it results in unbiased data collection.

The literature search produced 55 papers, dating from 1978 to 2018, which provided information on the thermal response curve for populations of all seven sea turtle species. Experimental design differed considerably among studies, and exclusion criteria were applied. Laboratory studies were excluded if i) they were back-switch experiments, where eggs were moved from one temperature to another during incubation; ii) eggs were experimentally manipulated using hormone application; or iii) more than one environmental condition was being manipulated. There is debate as to whether sampling dead-in-nest hatchlings during natural studies risks bias if a sex-specific survival rate exists (LeBlanc et al. 2012). As a study comparing the sex ratios

of nests using both dead-in-nest and euthanized hatchlings found similar pivotal temperatures between the two groups (dead in nest: 28.9 ± 1.9 °C, n = 298 offspring from 149 nests, euthanized: 28.9 ± 3.1 °C, n = 180 hatchlings from 19 nests) (LeBlanc et al. 2012), field studies relying on both euthanized and dead-in-nest hatchlings were retained. After applying exclusion criteria, 35 papers were retained, dating from 1978 to 2017. As some papers estimated the pivotal temperature for more than one species or location, we had 37 data-points.

Where possible (n = 31), we extracted raw data on the sex ratios produced at specific temperatures from the papers, and re-calculated the T_{piv} and TRT using the Hill equation within the R package embryogrowth (Girondot 1999). The Hill equation builds on the logistic equation by accounting for logarithmic scaling in the shape of the TRT (Fuentes et al. 2017). This approach allowed us to standardise TRT and define it as the range of temperatures at which between 5 and 95% female offspring are produced.

1.5.2 Quantifying local adaptation

Environmental data for the thermosensitive month of study clutches were accessed through the NOAA National Climatic Data Centre. As the environmental data is of monthly resolution, we considered the thermosensitive month of study clutches to be the month following oviposition. Studies varied in the reporting of their design, and so we made several assumptions; i) if the study was conducted across the season then we took the month after peak nesting as our thermosensitive period (n = 12); ii) if the year the study was conducted was not included within the study, we defined it as two years before publication date (n = 3). Mean air temperatures (1979 - 2014) were extracted from the NOAA Terrestrial Air Temperature V4.01 dataset at a spatial resolution of 0.5 degrees. Total monthly rainfall data (1979 – 2014) were extracted from the NOAA Climate Prediction Centre. The resolution of the data was too poor to provide

environmental data for some of the small islands where sea turtles nest, and so some locations (Ascension Island and Antigua) were omitted from environmental models.

We quality checked the data against average monthly values reported by weather websites. We removed a single outlier of a rookery in Columbia, as this study was conducted during an El Niño year (1998), and experienced extremely elevated temperatures and levels of precipitation. After this point was removed, we found that our data correlated strongly with online reports for average monthly conditions for both the thermosensitive month (temperature: $F_{1,35} = 11.923$, $p < 0.001$, precipitation: $F_{1,32} = 54.300$, $p < 0.001$) and the IPCC baseline (temperature: $F_{1,35} = 10.645$, $p < 0.001$, precipitation: $F_{1,35} = 122.13$, $p < 0.001$, Appendix 1, Figure A1.8).

Statistical analyses were performed using R (version 3.3.3). All models were backwards selected using AIC values to retain the most reduced model. Linear models were initially used to investigate the relationship of the T_{piv} and TRT with latitude. To quantify the effect of the local environment on the thermal response curve, we used mean monthly temperature, total monthly precipitation of the clutch thermosensitive month and their interaction as predictors, and both T_{piv} and TRT individually as response variables. Finally, to quantify the effect of local climate predictability on T_{piv} and TRT, we calculated the standard deviations of average temperature and total precipitation at rookeries between 1979 and 2014, and included these, and their interaction, as predictors in two linear models with TRT and T_{piv} as the responses. IPCC predictions were based on a baseline value of the mean temperature or precipitation between 1980 and 2000.

1.5.3 Estimating nest temperatures

Air and sand temperatures show a strong positive correlation (Laloë et al. 2014; Esteban et al. 2016), but this relationship is location specific, and will be influenced by factors

such as substrate type and sand albedo (Hays et al. 2001). We determined a heuristic, universal equation for predicting nest temperature based on available published data. First, we placed temperature loggers (TinyTag™), programmed to record temperature every 15 minutes, at the centre of 28 nests at our research site, in Boavista, Cape Verde, during the 2017 nesting season. We then correlated a daily average nest temperature from these data against the average daily air temperature extracted from www.worldweatheronline.com. This significant relationship ($F_{1,69} = 92.833$, $p < 0.001$, Appendix 1, Figure A1.4) was combined with similar air-sand temperature relationships from other studies (Esteban et al. 2016) (Appendix 1, Figure A1.5). We then regressed the combined air temperatures against sand/nest temperatures to determine a universal equation for nest temperature.

1.5.4 Future projections

Regional IPCC forecasts for temperature and rainfall between 2000 and 2100, during the thermosensitive month for each of the studied location were downloaded from the IPCC AR5 online archive. We selected projection models for two scenarios. RCP 2.6 assumes that stringent carbon emission control will constrain global warming to an increase between 0.7 and 1.5 °C, while RCP 6.0 predicts that warming will continue to increase after this point, likely exceeding 2 °C by the year 2100. We modelled three different scenarios for T_{piv} variation over this period; Scenario 1: We use a fixed T_{piv} of 29 °C to repeat past modelling studies (Laloë et al. 2014). Scenario 2: We modelled the case where T_{piv} can match environmental changes based on the determined correlation between T_{piv} and air temperatures as well as rainfall (Eq. 1). In scenario 3, we modelled a heritable T_{piv} that constrained the plastic response to 35.1% of its possible variation based on the assumption that T_{piv} can be a heritable trait as determined in painted turtles (McGaugh et al. 2011). For both Scenario 2 and 3, we incorporated the originally

reported T_{piv} at the appropriate year, and made all predictions in relation to this. TRT remained constant in all three models. Using our calculated nest temperatures and T_{piv}, we used the logistic equation described by Girondot (1999) to estimate sex ratio:

$$S_i = \left| \frac{R_i}{2\log\left(\frac{0.05}{0.95}\right)} \right| \qquad \ldots Eq.\ 3$$

$$Sr_i = 1 - \frac{1}{1 + e^{\frac{1}{S_i} * (K_i - \tau_i)}} \qquad \ldots Eq.\ 4$$

Where S is the rate of change, R is the TRT, Sr is the sex ratio, K is the T_{piv} and τ is the nest temperature.

OSRs of these cohorts were computed using the calculation from Laloë et al (2014), making the assumptions that i) males mate twice as frequently as females, ii) it takes 45 years for an individual to recruit to the reproductive population and iii) there is no difference in mortality rates between males and females:

$$OSR(t_{i+45}) = 100 \times \frac{Sr(t_i)_{females}}{Sr(t_i)_{females} + 2 \times Sr(t_i)_{males}} \qquad \ldots Eq\ 5$$

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Chapter 2. Maternally derived sex steroid hormones impact sex ratios of loggerhead sea turtles

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2.1 Abstract

An optimal sex ratio is arguably one of the most important traits of a species' demographics. Globally rising temperatures threaten species with temperature-dependent sex determination (TSD), by biasing sex ratios and altering population dynamics. Because sex steroid hormones can impact sex determination in TSD reptiles, variation in their maternal transfer within the egg yolk may form a mechanism to buffer the effects of temperature on sex ratio. We tested this hypothesis by quantifying the effect of maternal oestradiol (E_2) and testosterone (T) transfer on offspring sex in a threatened population of loggerhead sea turtles (*Caretta caretta*). Circulating levels of E_2 and T in nesting females, in egg yolks at oviposition and in hatchlings were measured. In a field experiment, we controlled for variation in incubation temperature by standardising the depth of nests relocated to an *in situ* hatchery. Using offspring hormone profiling, incubation duration and affinity propagation clustering, we estimated sex of individuals from these nests in a non-lethal manner, offering a novel conservation tool for this endangered species. Despite standardised temperatures, we found wide levels of variation in sex ratio, which showed a non-linear relationship with the ratio of E_2:T within in egg yolks. Hatchlings considered to be male are produced at equal levels of E_2 and T investment, with assumed females produced on either side of this optimum. Overall, maternally-derived hormones form a potential trans-generational mechanism of TSD plasticity that can modify offspring sex ratios in endangered sea turtles.

2.2 Introduction

Fifty years after the discovery of environmental sex determination (Charnier 1966), our understanding of its evolutionary significance, underlying mechanisms and ecological consequences in the light of environmental change remains incomplete (Ge et al. 2018; Warner & Shine 2008; Laloë et al. 2014; Mitchell & Janzen 2010). Many reptile and some fish species exhibit temperature-dependent sex determination (TSD), in which gonad differentiation is determined by temperature at a critical period of embryogenesis (Bull 1980; Deeming et al. 1988). Some species produce males at moderate temperatures and females at hot and cold extremes (e.g. the American alligator *Alligator mississippiensis* (Ferguson & Joanen 1983), Type II TSD), but, more commonly, TSD species produce an increasing proportion of a specific sex as temperatures rise (Type Ia: Males at low temperatures, e.g. the painted turtle *Chrysemys picta* (Bull & Vogt 1979); Type Ib: Females at low temperatures, e.g. the tuatara *Sphenodon punctatus* (Cree et al. 1995)). In all cases, both sexes are produced across a transitional range of temperatures (TRT), centered on a pivotal temperature at which both sexes develop in equal proportions (Yntema & Mrosovsky 1982). As a consequence of TSD, rising global temperatures present the potential for extreme sex ratio biases, with important implications for population dynamics (Hawkes et al. 2009; Witt et al. 2010; Laloë et al. 2014).

The adaptive value of TSD is still debated, but sex benefits under specific thermal environments are predicted by the widely favoured Charnov-Bull theory of differential fitness (Charnov & Bull 1977; Shine 1999). This has been demonstrated in eggs of the Jacky dragon (*Amphibolurus muricatus*) that were experimentally treated with an aromatase inhibitor, causing embryos to develop as males at female producing temperatures. These males showed lower lifetime reproductive success than controls (Warner & Shine 2008). While demonstrating the adaptive value of TSD, the use of an

aromatase inhibitor to manipulate sex in this study also highlights the role of sex steroid hormones on the TSD mechanism (Bowden et al. 2000; Elf 2003; Bowden & Paitz 2018).

Exogenous application of oestradiol (E_2) has been shown to feminise embryos from TSD species incubated at male-producing temperatures (Crews et al. 1991; Crews et al. 1989; Merchant-Larios et al. 1997; Wibbels et al. 1991b). In addition, the application of testosterone (T), the precursor androgen of E_2, can also feminise embryos via aromatase synthesis (Crews et al. 1989; Wibbels & Crews 1992), and the use of aromatase inhibitors can force the production of males at female-producing temperatures (Rhen & Lang 1995; Wibbels & Crews 1994). Both temperature and embryonic treatment with E_2 appear to activate the same molecular pathways, altering the transcription of the chromatin modifier gene *Kdm6b*, and conferring sensitivity to a key sex-determining gene, *Dmrt1* (Ge et al. 2018).

In two widely-studied TSD species exhibiting Type 1a TSD, the slider (*Trachemys scripta*) and the painted turtle, maternal transfer of sex steroid hormones into eggs varies seasonally (Bowden et al. 2000; Carter et al. 2017). In these species, elevated concentrations of yolk E_2 and an increasing E_2:T ratio increase the likelihood of feminisation at a given temperature, effectively reducing the pivotal temperature of a clutch (Bowden et al. 2000; Carter et al. 2017). Should these patterns be found in wild systems, variation in maternal hormone transfer to eggs may be a universal mechanism to (i) change the threshold at which temperature affects an individual's sex, (ii) modify the sex ratio of the entire clutch, and (iii) buffer against the effects of rapid global temperature changes on sex ratios for TSD species.

There is a particular need to understand the impacts of climate change on the demographics of threatened species. For species such as sea turtles, advances in understanding TSD mechanisms have been constrained by a lack of non-lethal methods

to sex hatchlings (Wyneken et al. 2007). This issue is especially important for endangered populations, where sacrificing individuals is not an option (Wyneken et al. 2007). As a consequence of rising temperatures, extreme feminisation of sea turtle populations by the end of the century has been forecast (Hawkes et al. 2009; Laloë et al. 2014; Jensen et al. 2017; Tanner et al. 2019). Some studies already suggest effects of offspring sex ratio bias are visible in adult populations (Jensen et al. 2017). Yet, these predictions of population dynamics in the face of climate change assume a fixed pivotal temperature with no account for physiological mechanisms that may increase variation in this trait.

Here, we standardised the thermal environment of relocated loggerhead turtle (*Caretta caretta*) clutches, and explored the role of maternal transfer of E_2 and T in the sex determination process, in a field experiment conducted in the Cape Verde archipelago. If temperature is the sole driver of sex determination, under a standardised thermal environment we would expect to observe equal sex ratios among clutches. If inter-clutch variation exists, it would emerge from intrinsic characteristics of the eggs, such as maternally derived hormones. To test this scenario, we quantified E_2 and T concentrations in maternal plasma, egg yolks and hatchling plasma. We developed a non-lethal method to estimate hatchling sex using individual hormone profiles, in order to determine the sex ratio of the relocated clutches. Observed inter-clutch variation in sex ratio correlated with yolk hormone concentrations. Finally, we illustrate how maternal hormone transfer might impact sex ratio in the face of IPCC climate change predictions, by parameterising a simple mathematical model that refines future population dynamics.

2.3 Results

Using ELISA assays, we first quantified concentrations of the sex steroid hormones E_2 and T in both the blood plasma of 26 nesting females and up to two of their eggs directly after oviposition. High levels of individual variation were observed in adult plasma hormone levels (Appendix 1, Table A2.1), with a mean T concentration of 1148.48 ± 148.63 (SE) pg/ml, a mean E_2 concentration of 235.79 ± 22.71 (SE) pg/ml, and a mean E_2:T ratio of 0.32 ± 0.05 (SE). Linear models (LM) showed positive correlations between E_2 and T in both female plasma (Appendix 2, Figure A2.1A, $F_{1,16}$ = 4.608, p = 0.048) and egg yolks (Appendix 2, Figure A2.1B, $F_{1,23}$ = 7.338, p = 0.013). In reptiles, maternally derived hormones have been shown to be constant across all eggs of a given clutch (Janzen et al. 1998), and we confirmed this with a subset of clutches where two egg yolks were analysed (Paired t-tests: T: df = 11, t = 0.224, p = 0.827; E_2: df = 10, t = -0.885, p = 0.397; E_2:T: df = 9, t = -1.173, p = 0.271).

There was a significant parabolic relationship between T concentrations in adult plasma and egg yolks (Figure 2.1A: LM: $F_{1,14}$ = 5.263, p = 0.038), where concentrations of yolk T were lowest in eggs originating from females with intermediate levels of plasma T, but did not correlate with clutch size (Figure 2.1D: $F_{1,14}$ = 0.032, p = 0.862). In contrast, adult female plasma E_2 levels were not correlated with E_2 concentrations found within their egg yolk (Figure 2.1B: LM: $F_{1,21}$ = 0.908, p = 0.351), but as clutch size increased, yolk E_2 concentrations significantly decreased (Figure 2.1E: LM: $F_{1,21}$ = 4.945, p = 0.037). The maternal E_2:T ratio showed a non-linear relationship with the E_2:T ratio in the egg yolk (Figure 2.1C: $F_{1,14}$ = 6.493, p = 0.023), but was not correlated with clutch size (Figure 2.1F: $F_{1,14}$ = 1.682, p = 0.215).

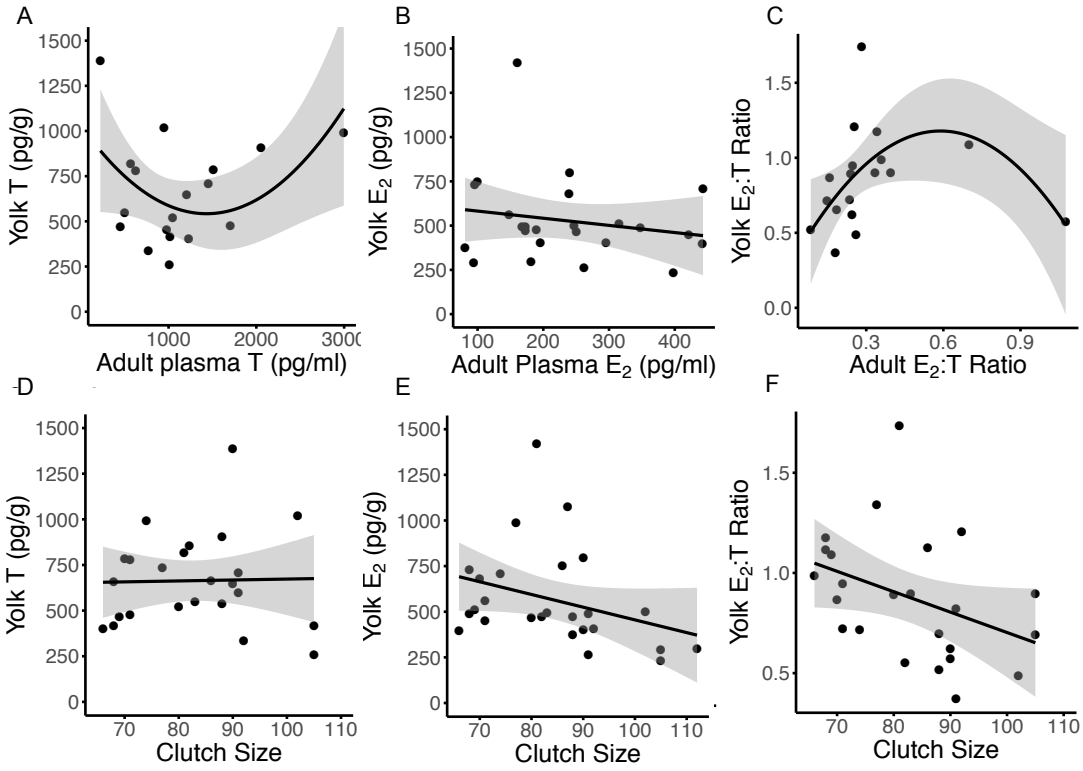

Fig. 2.1: Scatterplots showing the relationship between adult circulating hormone concentrations and clutch size on yolk hormone concentrations. A) Yolk T has a significant, non-linear relationship with circulating adult T ($F_{1,14}$ = 5.263, p = 0.038); B) There is no relationship between Yolk E_2 and the concentrations found in circulating adult plasma ($F_{1,21}$ = 0.908, p = 0.351); C) There is a non-linear relationship between the E_2:T ratio found within the yolk, and that within adult female plasma ($F_{1,14}$ = 6.493, p = 0.023) D) Clutch size has no effect on yolk T concentrations ($F_{1,14}$ = 0.032, p = 0.862); E) As clutch size increases, concentrations of E_2 within the yolk decrease ($F_{1,21}$ = 4.945, p = 0.037); F) As clutch size increases, the yolk E_2:T ratio decreases ($F_{1,14}$ = 1.682, p = 0.215).

Immediately after oviposition, the clutches of these 26 females and two others (n = 28) were relocated into an *in-situ* experimental hatchery that was protected from predation, yet exposed to natural sand and weather conditions. We recorded the number of eggs in each clutch (hereafter clutch size), and buried clutches at a depth of 55 cm to standardise the thermal incubation environment. We confirmed the standardised thermal environment using data loggers placed at the centre of the clutch (mean thermosensitive period temperature = 30.02 ± 0.05 (SE) °C, Appendix 2, Figure A2.2). While the thermal environment of individual eggs within a clutch will vary slightly due to their position in the nest, the standardised depth of the bottom eggs ensures that temperatures gradients were similar across all clutches. The small amount of between-clutch temperature variation observed was explained by differences in clutch size ($F_{1,26}$ = 4.418, p = 0.045), resulting from metabolic heat produced by developing embryos (DeGregorio & Williard 2011). Assuming the pivotal temperature to be 29 °C, as has previously been used for this population (Laloë et al. 2014), we would predict that this incubation temperature would produce 14.35 ± 0.01 (SE) % male offspring if temperature was the sole determinant of sex ratio (Figure 2.2A). Incubation duration, the time between oviposition and hatchling emergence, is also often used as a proxy to predict offspring sex ratios and was recorded for each clutch (Mrosovsky et al. 1999). Using the established logistic relationship between incubation duration and sex ratio observed in loggerhead turtles from Kyparissia, Greece (Figure 2.2B, the closest location where the relationship between incubation duration and offspring sex ratio has been quantified), the predicted sex ratio of our study clutches would be 47.5 ± 6 (SE) % males (Mrosovsky et al. 2002). This suggests that levels of sex ratio variation are far greater than those we would expect from temperature alone.

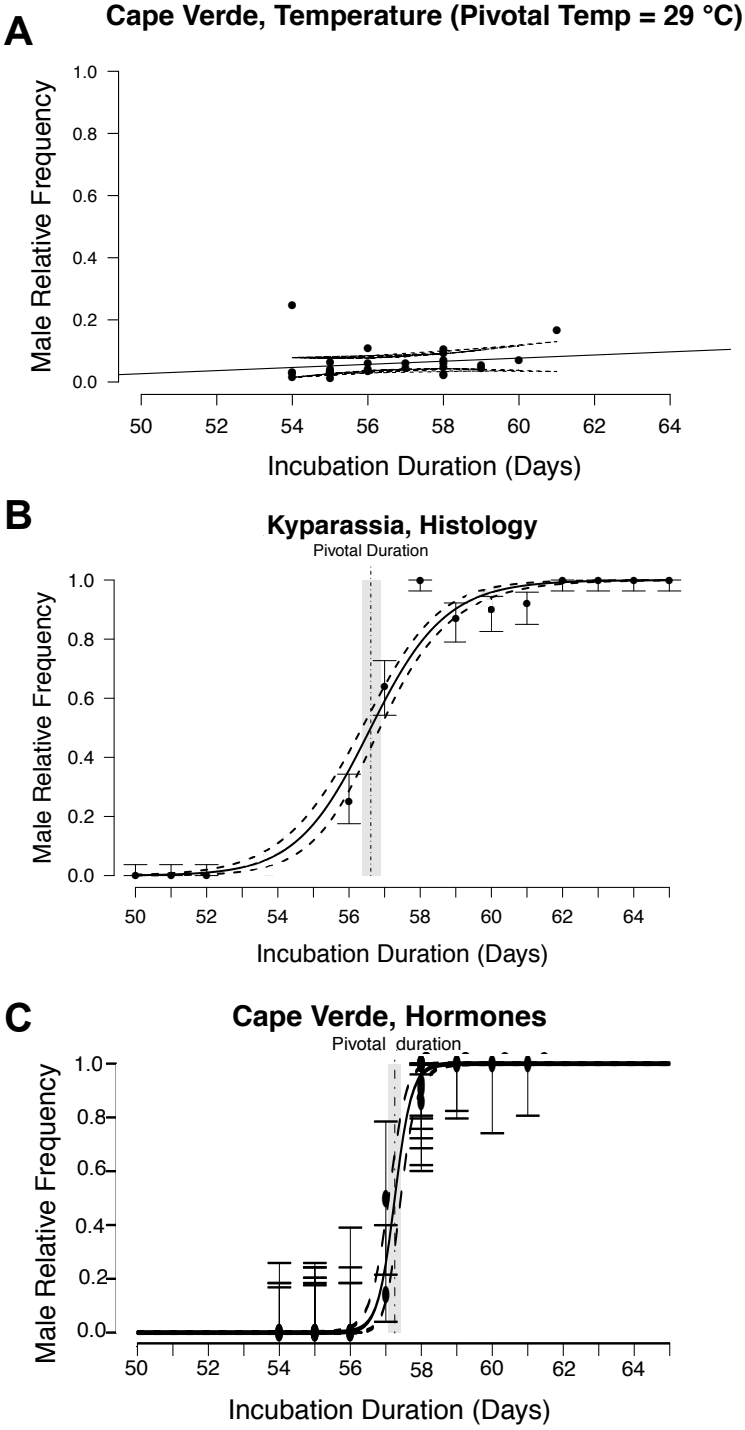

Figure 2.2: Scatterplots (with regression lines and 95% confidence intervals) showing ratios of study clutches A) as would be expected with a pivotal temperature of 29 °C B) based on the relationship between incubation duration and clutch sex ratios in Greece (Mrosovsky et al. 2002) and C) as determined by hormone profiles and machine learning algorithm of individual offspring.

While the incubation duration is a widely-used proxy for estimating the sex ratio of sea turtle offspring, currently the only accurate method to resolve individual sex requires sacrificing hatchlings and histological examination - a limiting factor for endangered populations (Mrosovsky & Benabib 1990; Ceriani & Wyneken 2008). However, we developed a possible new method to estimate individual sex without the need to sacrifice animals. After taking 100 – 150 µl of blood from 365 offspring from 28 clutches after emergence (mean offspring per clutch = 13 ± 4), we measured plasma hormone concentrations using ELISA assays. Hatchling hormone levels varied among individuals (Appendix 1, Table A2.1) and among clutches, with the average E_2:T ratio of clutches ranging from 1.06 ± 0.13 (SE) to 3.56 ± 0.68 (SE). We used affinity propagation clustering (APC) on hatchling E_2:T ratios guided by incubation duration to identify clusters of individuals with a similar hormonal phenotype. APC iteratively considers the similarity of a data point to its neighbours. Importantly, it does not require the number of possible clusters to be defined *a priori,* as is necessary for other clustering approaches such as k-means (Frey & Dueck 2007). We identified three APC clusters (Figure 2.3A). Two of these originate from clutches with short incubation durations, the classic trait of females, and were distinguished by differences in their mean E_2:T ratio (Appendix 2, Figure A2.3, Cluster 1: mean = 4.45 ± 0.26 (SE), Cluster 2: mean = 1.72 ± 0.05 (SE), t-test: df = 44.08, t = 10.273, $p < 0.001$). The third group is formed by individuals from clutches with longer incubation durations (t-test: df = 299.3, t = -32.933, $p < 0.001$) and a low E_2:T ratio (Appendix 2, Figure A2.3, mean = 1.52 ± 0.06 (SE)), the characteristics of male sea turtles.

While we were unable to verify these results directly using histology, several positive theoretical controls were used to confirm this method. Importantly, as our APC method combines incubation duration with individual hormone concentrations, we were unable to define population-level thresholds for estimating male/female hatchlings using either

E_2 or T in isolation (Appendix 2, Figure A2.4). Instead, a nest-specific threshold E_2:T ratio appears to differentiate between hatchlings we have estimated as male/female from nests that are predicted to be mixed sex (Appendix 2, Figure A2.5). Despite the lack of definitive population-level hormone thresholds to define sex, linear mixed effect models (LMM) using clutch ID as a random factor revealed significant differences in hormone levels between the two estimated sexes, that were directly comparable to previous studies in which individuals' sex was positively confirmed through histology (Xia et al. 2011; Gross et al. 1995). T levels were higher overall in hatchlings estimated to be males (Figure 2.3Bi, LMM: $F_{1,60} = 10.673$, $p = 0.002$, mean = 63.63 ± 2.89 (SE) pg/ml) than in those estimated to females (mean = 52.54 ± 2.34 (SE) pg/ml), and conversely E_2 levels were higher in probable females (Figure 2.3Bii, LMM: $F_{1,57} = 7.521$, $p = 0.008$, mean = 92.94 ± 3.06 (SE) pg/ml) than in probable males (mean = 81.66 ± 3.16 (SE) pg/ml), as was the overall E_2:T ratio (Figure 2.3Biii, LMM: $F_{1,48} = 28.652$, $p < 0.001$, probable females: mean = 2.22 ± 0.09 (SE); probable males: mean = 1.52 ± 0.06 (SE)). LMMs did not detect any difference in mass ($F_{1, 348} = 0.024$, $p = 0.878$) or size ($F_{1, 218} = 0.766$, $p = 0.382$) between the sexes, as would be expected under these conditions by the Charnov-Bull theory (Charnov & Bull 1977, but see Booth 2017). Second, by combining individual offspring sex into an estimate of clutch sex ratio, and comparing this to the incubation duration, we found the specific logistic regression curve that characterises incubation durations in Type Ia TSD species (Figure 2.2C). The pivotal duration was fitted to a value of 57.25 days (95% CIs: 57.09, 57.43), with a transitional range of incubation durations of 2.15 days (95% CIs: 1.52, 2.77).

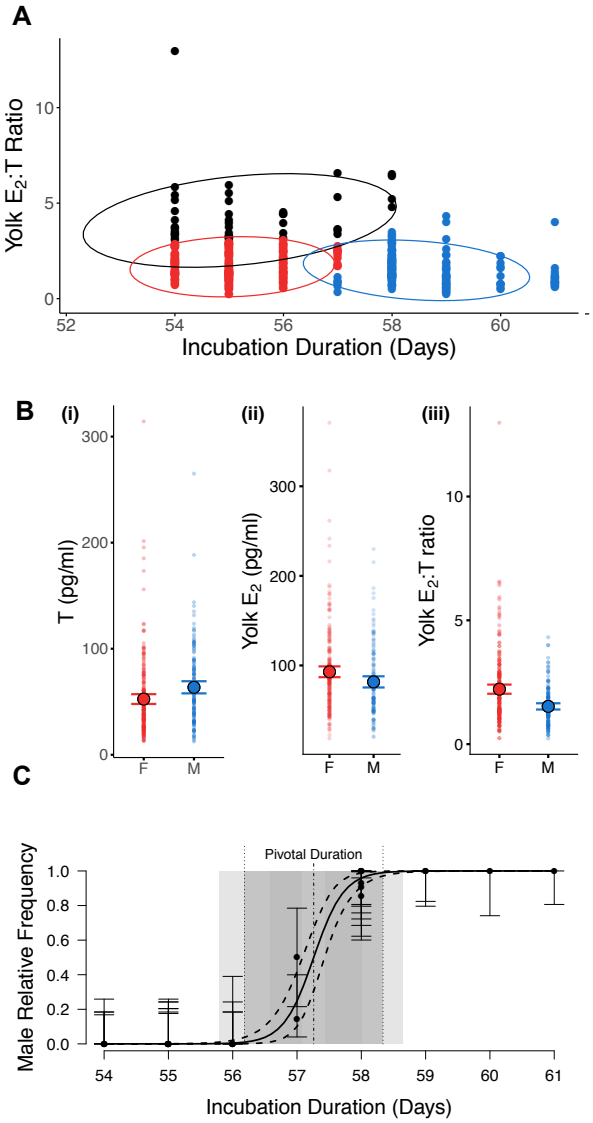

Figure 2.3: Individual sex as estimated by affinity propagation clustering (APC). A) Scatterplot of hatchling E_2:T ratios against clutch incubation duration. APC identifies three different clusters, equating to estimates of female (red and black) and male (blue) offspring; B) Significant differences in the concentrations of T ($F_{1,60} = 10.673$, $p = 0.002$), E_2 ($F_{1,57} = 7.521$, $p = 0.008$) and the E_2:T ratio ($F_{1,48} = 28.652$, $p < 0.001$) between estimated male and female offspring shown by using the mean and 95% confidence intervals; C) Scatterplot showing frequency of estimated male offspring estimated by APC in relation to incubation duration. The pivotal duration was estimated at 57.25 days.

Importantly, if individual sex were incorrectly assigned, this distinctive pattern of TSD species would not be seen. This method estimates that 40.49 ± 8.98 (SE) % male offspring were produced (Figure 2.2C). This suggests 26.1% more males and far more variation in clutch sex ratio than would be expected based on incubation temperatures alone. Our estimate is slightly below (7.1%) that estimated from parameters based on incubation durations in Kyparissia, suggesting population differences in development rate, likely as a result of different average pivotal temperatures between rookeries.

After establishing that inter-clutch variation in sex ratio (and also in incubation duration, see SI) was too great to be produced by temperature alone, we tested whether metabolic heat and/or maternal hormone transfer in the yolk predicted incubation duration and the estimated sex ratio. Yolk T correlated negatively with both incubation duration (LM, $F_{1,22} = 10.624$, $p = 0.003$) and the proportion of probable males produced within a clutch (Figure 2.4A, Binomial generalised linear mixed effect models (GLMM), $x^2 = 4.371$, df = 1, $p = 0.037$), but metabolic heat had no effect (incubation duration model: $F_{1,22} = 2.436$, $p = 0.133$, sex ratio model: $x^2 = 2.111$, df = 1, $p = 0.146$). There was no relationship between yolk E_2 and incubation duration or estimated clutch sex ratio (Figure 2.4B, incubation duration: $F_{1,23} = 3.169$, $p = 0.088$, sex ratio: $x^2 = 0.183$, df = 1, $p = 0.669$), yet the yolk E_2:T ratio showed a non-linear relationship with both incubation duration (Figure 2.4C, $F_{1,21} = 12.882$, $p = 0.002$) and estimated sex ratio independently of temperature ($x^2 = 7.064$, df = 2, $p = 0.029$). A maximum incubation duration of 57.2 days was observed at an equal hormone ratio (E_2:T of 1.05, $y = -7.8x^2 + 16.3x + 48.7$) with the highest levels of male offspring developing at this point.

The production of hatchlings estimated to be male was highest when maternal investment of E_2 and T to the yolk is equal. Asking whether the production of either sex is more costly in terms of total hormone investment, we compared the total hormone concentration (E_2 + T) with the overall E_2:T ratio. This relationship was again non-

linear, with total hormone investment being highest when the E_2:T ratio is unequal (LM: $F_{2,22} = 4.951$, $p = 0.017$), which would suggest that female production possibly requires more maternal investment than males. The total hormone investment also showed a non-linear relationship with clutch size ($\log(E_2 + T)$: $F_{2,22} = 4.306$, $p = 0.026$), with an initial increase in investment across clutch sizes between 65 and 75 eggs, after which investment declined with increasing clutch size.

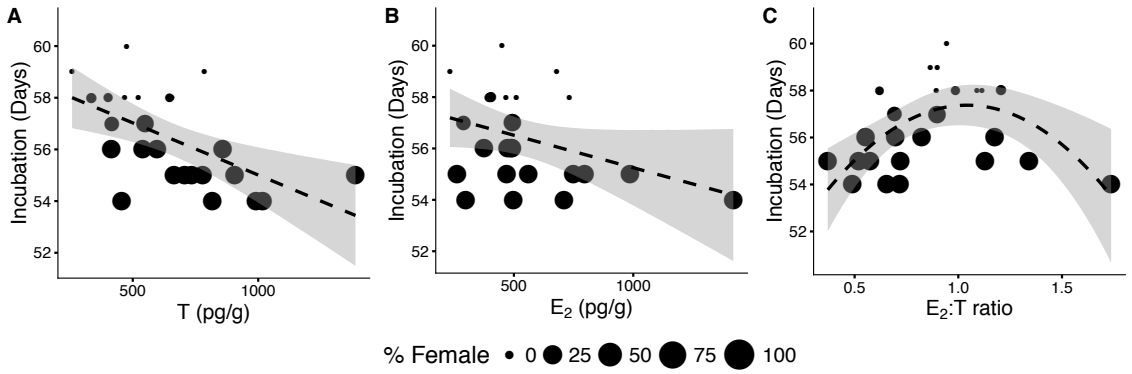

Figure 2.4: Scatterplots showing relationship between maternally derived T ($F_{1,22} = 10.624$, $p = 0.003$), E_2 ($F_{1,23} = 3.169$, $p = 0.088$) and E_2:T ratio ($F_{1,21} = 12.882$, $p = 0.002$) concentrations within egg yolks, with incubation duration. Size of data points relates to the sex ratio as estimated by APC.

Finally, to illustrate how maternal hormone transfer could impact population dynamics, we performed mathematical modelling of hatchling sex ratios for the Cape Verde population based on IPCC climate emission prediction SRES2, from the Fourth Assessment Report released in 2007, as conducted in a previous study (Laloë et al. 2014). We made the simple assumption that the effect of maternally derived hormones on sex ratio remains the same across a thermal gradient and applied the 26.1% observed difference in male offspring production for the coming century (Figure 2.5). With a mechanism of this possible strength, the population would be unlikely to reach the

levels of extreme feminisation previously forecasted. While the projection will not be entirely accurate, as it remains to be determined how maternal hormone transfer interacts with different incubation temperatures, this model illustrates the possible importance of trans-generational hormone transfer for population dynamics.

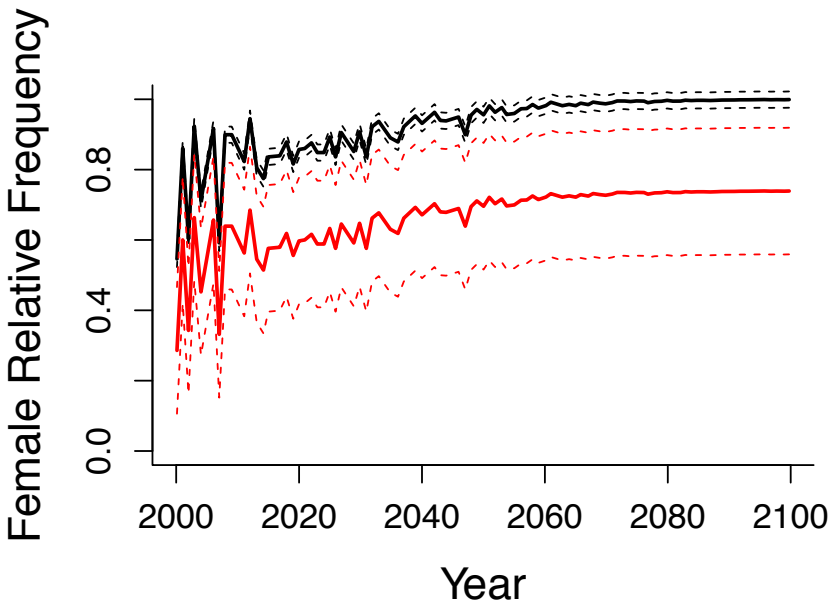

Figure 2.5: Time series showing the estimated population sex ratio of Cape Verde over the next century if it was determined by temperature alone (Laloë et al. 2014) (black) and incorporating the effect of hormones observed here on the sex determining mechanism (red) along with the 95% confidence intervals.

2.4 Discussion

Given the many considerable historical climate shifts experienced by TSD species, they are likely to have evolved mechanisms to avoid unviable biases in offspring sex ratio (Mitchell & Janzen 2010). By experimentally standardising the thermal environment of loggerhead sea turtle nests *in-situ*, we investigated whether maternally derived hormones correlate with offspring sex independently of temperature. First, building on

past literature, we developed a non-lethal method to estimate hatchling sex upon emergence from nests, using a machine learning clustering algorithm which considers individual circulatory sex steroid hormones in relation to their incubation duration. We used this to find a non-linear correlation between the clutch sex ratios and the ratio of maternally derived E_2:T within the egg yolk. Equal investment in both hormones at low concentrations within the yolk appears to maximise the production of offspring we predicted to be male, while increasing the concentration of either E_2 or T, along with overall hormone investment, likely feminises clutches. We demonstrate the theoretical influence that this trans-generational mechanism may have on offspring sex ratio biases that are forecast to emerge from rapidly rising temperatures.

To date, the inability to determine hatchling sex non-lethally has constrained the study of TSD mechanisms in sea turtles. A clustering approach that identifies individuals with similar phenotypes (here hormone profiles) that match control traits of male and female offspring (incubation duration) overcame this problem. Using E_2:T thresholds to define hatchling sex has been verified with histological analysis in loggerhead (Gross et al. 1995) and green (Xia et al. 2011) turtles, but as E_2:T levels vary considerably between clutches, it is difficult to delineate accurate population-level thresholds. Using an APC method guided by incubation duration to group hormone profiles, a common proxy for sex ratio, we avoided the need to define thresholds and the need to sacrifice individuals (Mrosovsky et al. 1999). Although we were unable to verify this method using histology, there is evidence for the reliability of this method, as (i) circulating E_2:T ratios of male and female offspring identified in this study and (ii) pivotal duration, both match those reported in studies where sex has been confirmed with histology (Kaska et al. 2006; Mrosovsky et al. 2002; Gross et al. 1995; Marcovaldi et al. 1997; Xia et al. 2011), and (iii) the relationship between sex ratio and incubation duration fits the known logistic regression curve observed in Type Ia TSD species. We hope that in time,

this non-lethal approach could make a useful contribution to research on TSD in sea turtle species and also to wider conservation efforts.

Despite the standardised thermal environment of clutches within this experiment, high levels of variation in incubation duration and estimated sex ratio were observed between nests, which correlated with maternally derived hormones within the egg. The relationship between the yolk E_2:T ratio and clutch sex ratio was best described by a quadratic curve, centred on an equal concentration of both hormones and ranging from 0.37 to 1.73. When maternal investment of E_2 and T was equal but low, incubation durations were long, and hatchlings that we estimated to be male were produced. If hormone investment was skewed in either direction, estimated sex ratios appear to become increasingly feminised. The effects of elevated levels of both E_2 and T on sex ratio in this study are consistent with experimental manipulation of these hormones in other TSD species (Crews et al. 1989; Wibbels et al. 1991b; Ge et al. 2018). Interestingly, in all other reptiles for which data is available, the E_2:T ratios that are transferred to the yolk consistently remain below or above the ratio of one (Radder 2007). Thus, to our knowledge, our study appears to be unique in finding that both hormones potentially influence the feminisation process of reptiles under natural conditions.

Overall hormone concentrations within the yolk were lowest at an equal E_2:T ratio. If this ratio departed from one in either direction, total concentrations of yolk hormones increased. As E_2 and T positively correlate within the egg, if investment in either hormone is elevated, there is an associated increase in the other. We theorise that outcome of this is that greater investment would be required to skew E_2:T ratios in a manner that would favour the production of female offspring. At a low temperature, if E_2:T ratios are skewed, and total hormone concentrations are high, feminisation could be achieved through either the presence of E_2 directly, or by the synthesis of E_2 from its

precursor, T, by the aromatase enzyme. Conversely at the same temperature, if E_2 and T are in equilibrium, low concentrations of E_2 do not appear to be sufficient to feminise the clutch. However, product-feedback inhibition likely prevents further E_2 being synthesised from T, and consequently male offspring are produced.

There is no doubt that temperature is the primary determinant of sex in TSD species, yet there is growing evidence that E_2 and, indirectly, T affect the same developmental pathways (Ge et al. 2018; Wibbels et al. 1991b). Accordingly, it is possible that a clutch specific threshold exists for feminisation that is the product of both temperature and maternal hormone transfer. Under this scenario, a shift towards an equal E_2:T ratio and lower maternal investment could increase the pivotal temperature away from the feminisation threshold, and consequently warmer temperatures would be required to feminise a clutch. This aligns with sex ratios observed here, which were estimated to contain 26.1% more males than expected from a pivotal temperature of 29 °C. However, this mechanism will probably be constrained by physiological limits of maternal hormone investment.

Two maternal traits show a relationship with levels of hormone transfer to the clutch. Firstly, T concentrations within the yolk correlated non-linearly with those in maternal plasma. Disentangling the cause of such a relationship is complex as it is likely to result from multiple physiological cascades, and attempts to accurately explain this are beyond the scope of this study (Groothuis & Schwabl 2008). However, this relationship does allow us to link T investment to maternal state. Should maternal T vary in response to environmental cues, as in the spined toad, *Bufo spinosus,* it could allow individuals to plastically match feminisation thresholds to temperature variation, and maintain more constant sex ratios across a nesting season (Brischoux et al. 2018). Indeed, differences in the yolk E_2:T ratio transferred within a population of painted turtles resulted in a seasonal shift of the pivotal temperature as environmental conditions changed (Bowden

et al. 2000). Secondly, total E_2 investment within eggs decreased as clutch size increased, and total hormone concentrations were low in large clutches. Thus, in large clutches with more metabolic heat production, the threshold for feminisation will be high. We propose from these results that there are two distinct mechanisms that can affect the ratio of E_2:T within the yolk, which explains how elevated investment in either hormone may lead to feminisation. There is considerable variation in circulating T and E_2 levels between sea turtle populations and species (Appendix 2, Table A2.1), which could suggest an element of local adaptation in response to environmental conditions, and a heritable component to baseline levels (Tschirren et al. 2009).

Overall, our work highlights a previously under-considered physiological mechanism for individual variation in the TSD process within sea turtle species. There is a need for management plans that use temperature-based models to predict future sex ratios to account for maternal hormonal influence, as this will have considerable implications for population dynamics.

2.5 Methods

2.5.1 Sample Collection

We studied nesting loggerhead sea turtles on the island of Boavista, part of the Cape Verde archipelago in the eastern Atlantic. The sampling site (15°58'18.6"N, 22°48'06.2"W) is a 400 m stretch of coastline on the southern tip of this island. Twenty-eight nesting females were sampled between 17 July and 1 August 2017. Immediately after oviposition, females were individually marked with PIT (AVID) and metal (Inconel) tags (Stiebens et al. 2013). Blood samples of 1-4 ml in volume were collected from the dorsal cervical sinus of 26 females using a 40 mm, 21-gauge needle and 5 ml syringe, and stored within lithium heparin containers. Finally, curved carapace length (CCL) and width (CCW) were measured (± 0.5 cm).

The clutches of these turtles (containing 83 ± 3 (SE) eggs) were relocated to an experimental hatchery protected from predation, situated on the nesting beach. At this point, up to two eggs from the 28 clutches were removed from each clutch for yolk hormone analysis, and the rest of the clutch was buried at a depth of 55 cm. By using a standard depth, temperature was controlled for, whilst maintaining an otherwise natural environment. A TinyTag™ temperature logger was placed at the centre of each clutch, programmed to take a reading every 15 minutes throughout the incubation period (accuracy ± 0.2 °C). As anticipated, the uniform depth standardised the incubation temperature of the nests to 30.05 ± 0.05 (SE) °C during the middle third of incubation, the period where embryo sex is typically established (Mrosovsky & Pieau 1991; Wibbels et al. 1991a; Crews et al. 1994). This variation in temperature is extremely conserved, and is representative of the thermal variation produced within treatments under controlled laboratory incubations (Mrosovsky et al. 1992; Mrosovsky et al. 2009). Upon emergence, twenty hatchlings were randomly selected for blood sampling (100 – 150 µl) from the dorsal cervical sinus, using a 26-gauge needle and 1 ml syringe (Wibbels et al. 1998). Samples were stored within lithium heparin coated tubes. Notch-to-notch straight carapace length (SCL) and, width (SCW) were measured using digital callipers (± 0.01 mm), and mass was measured with a digital scale (± 0.1 g)).

The blood samples of both the adults and offspring were refrigerated for up to 48 h before being centrifuged to extract plasma. Egg yolks were separated from the albumen, and all samples were stored at -20 °C until extraction.

2.5.2 Hormone extraction

Commercially available ELISA kits for both E_2 (Catalogue # ADI-900-174, ENZO Life Sciences) and T (Catalogue # ADI-900-065) were used to measure steroid levels in all samples. Details for hormone extraction protocols are given in Appendix 2. Not all

blood samples had sufficient volume for hormone extraction. Consequently, we extracted E_2 from 24 adults and 388 hatchling blood samples, and T from 19 adult and 367 hatchling blood samples. This provided us with E_2:T ratios for 18 adult females, and 365 hatchlings. E_2, and T were successfully extracted from the yolks of 26 out of the 28 sampled clutches.

2.5.3 Statistical Analyses

All analyses were conducted with R 3.3.3, using the R packages *lme4* and *lmerTest* for fitting linear mixed models (LMMs) and generalized linear mixed models (GLMMs). A paired t-test was used to compare intra-clutch E_2 and T levels between two eggs in a subset of clutches (n = 13), to test whether that there was variation in hormone investment between eggs in a clutch. As there was no difference between eggs from the same clutch, for subsequent analyses the average hormone was used where possible, while a single egg was used for the remainder of the clutches. Correlations between E_2 and T in female plasma and yolks, the effect of clutch size on temperature, and the effects of metabolic heat and maternally derived hormones on incubation duration were tested using general linear models (LM). A non-linear relationship between the E_2:T ratio on incubation duration was fitted using a quadratic curve. Similarly, when considering the relationship between E_2:T and total hormone investment, we also fit a quadratic model. LMs were also used to estimate the correlation of clutch size and plasma hormone concentrations with yolk hormone concentrations.

We used Algorithm Propagation Clustering (APC, Frey & Dueck 2007) to identify individual sex. Cluster assignment was made based on the plasma E_2:T ratio of hatchlings, guided by their incubation duration. Determining hatchling sea turtle sex using the E_2:T ratio has previously been extremely accurate (96% and 96.7% respectively) for artificially incubated eggs of loggerhead and green sea turtles (Gross et

al. 1995; Xia et al. 2011) that were ultimately sacrificed for verification. Since variation likely exists among rookeries, those thresholds however cannot be blindly applied to new populations. T-tests were used to compare hormone levels of putative male and female hatchlings. A response curve of these estimated sex ratios to incubation duration was produced using the logistic equation function of the R package *embryogrowth* to further verify the accuracy of our non-lethal sexing method.

After estimating the sex of individuals, LMMs were used to compare individual size and mass between the sexes and the APC clusters. Finally, we used binomial GLMMs to determine whether individual hatchling sex was predicted by maternal hormone investment or temperature. For all LMM and GLMM analyses, clutch was included as a random factor to account for individual variation. Model selection was based on AIC criteria, using a likelihood ratio tests to select for the best models. P-values of the selected models were obtained by with the *car* R package, and models were verified for over-dispersion.

Thermal estimates of sex ratio were calculated using the equation first presented by Girondot in 1999, under an assumed pivotal temperature of 29 °C. Estimates of sex ratio based on incubation duration were made based on data from a study on a neighbouring loggerhead sea turtle population that nests in Kyparissia, Greece, and was confirmed with histology (Mrosovsky et al. 2002). To generate an illustrative model that compared the results of our study with future predictions based on temperature alone, we extracted data from a previously published study predicting sex ratios until 2100 based on temperature alone. We then compared our observed mean clutch sex ratio to that expected from a pivotal temperature of 29 °C, and added the difference, along with 95% confidence intervals, to the original prediction.

2.6 Acknowledgements

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Chapter 3: Impacts of parasite infection on loggerhead sea turtle feeding ecology and reproductive success.

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Keywords: Parasites, trade-off, immunity, sea turtle, bet-hedging, terminal investment

3.1 Abstract

Long-term monitoring of the effects of parasites on endangered host species provides an opportunity to study the consequences of infection on host fitness and population dynamics. Such studies are particularly important in the face of rapid climate change, which threatens to disrupt host-parasite interactions with unknown consequences. In a nine-year long study of the endangered loggerhead sea turtle (*Caretta caretta*) population nesting in the Cape Verde archipelago, we describe the spatiotemporal variation of *Ozobranchus margoi*, a sanguivorous leech best known as a vector for the sea turtle fibropapilloma virus. We quantified its association with turtle feeding ecology using stable isotopes, and measured the influence of infection on reproduction. We found that the prevalence of this parasite has increased since 2010, and stable isotope analysis of host skin samples suggests transmission occurs within the host's feeding grounds. Interestingly, we found a significant interaction of turtle size and infection on reproductive fitness of turtles. Small and infected females produce fewer offspring of poorer condition, while in contrast, the largest infected turtles produce large clutch sizes and large offspring. We interpret this interaction as possible evidence for a size-dependent shift in reproductive strategy from bet hedging to terminal investment, upon infection. This link between infection and reproduction underscores the importance of using long term monitoring to quantify the impact of disease dynamics over time.

3.2 Introduction

Host-parasite interactions are sensitive to environmental perturbations, and as climate change impacts biodiversity and ecosystem functioning, the risk of disruption to these evolutionary-tuned dynamics increases (Brooks & Hoberg 2007; Brunner & Eizaguirre 2016; Sala et al. 2000; Tompkins et al. 2011; Bellard et al. 2012; Hooper et al. 2012). Long-term monitoring of host-parasite systems serves as a tool to detect slight changes in dynamics before major ecosystem shifts occur. For instance, a nine year-long survey of avian malaria (*Plasmodium circumflexum*) in blue tits (*Cyanistes caeruleus)* revealed oscillations in disease transmission among years that were driven by changes in host proximity to water (Lachish et al. 2011A). Acute infection of this disease causes significant host mortality (Lachish et al. 2011B). Similarly, the effects of pathogens on evolutionary dynamics were elucidated in another long-term study that showed parasite infection (*Teladorsagia circumcincta*) resulted in selection against inbreeding within a small population of Soay sheep *Ovis aries*, as infected individuals with low genome-wide heterozygosity had lower fitness (Coltman et al. 1999). Because the outcomes of environmental change on disease transmission and virulence remain difficult to predict (as disease transmission may be elevated eg: Harvell et al. 2002; Yang Xie et al. 2016; or reduced eg: Goedknegt et al. 2015; Gehman et al. 2018), there is a need to measure empirically how parasites impact populations, particularly those that are already threatened by extinction. In this sense, the finely tuned interactions between hosts and their parasites serve as a magnifying glass with which to measure the effects of environmental change on populations before slower ecosystem shifts happen.

Deterministic models suggest that parasites should become extinct before their host, however there are exceptions: frequency-dependent transmission, stochastic extinction at low densities, and biotic or abiotic reservoirs for parasites may all drive the extinction of vulnerable host populations (De Castro & Bolker 2005). A notorious example is that

of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, responsible for the decline of 6.5% of amphibian species and at least 90 presumed extinctions worldwide (Scheele et al. 2019). Its ecological success has been linked to increasingly favourable environmental conditions resulting from climate change (Scheele et al. 2019). While direct mortality is of particular concern in the case of the chytrid fungus, indirect effects of infection on host condition, such as fecundity and life history, may also have significant long lasting effects on host population dynamics (McCallum & Dobson 1995; Godwin et al. 2015).

An efficient feeding strategy is arguably one of the most important life history traits of an individual, maintaining nutrient uptake, physiology and reproduction (Werner & Anholt 1993; Naef-Daenzer & Kellert 1999; Simpson et al. 2004). In extreme cases, parasites and pathogens may prevent feeding of their host. Fibropapillomatosis in sea turtles leads to cutaneous lesions on soft tissues, that impair vision, locomotion and eating capacity before death (Herbst 1994). In less severe cases, an ecological impact of infection can be detected by a change in a host's trophic niche (Médoc et al. 2011; Pegg et al. 2015; Britton & Andreou 2016). Feeding rate is commonly influenced by infection, with hosts often increasing levels of food consumption to meet the energetic demand of mounting an immune response (Povey et al. 2008; Brunner et al. 2017). Alternatively, in some cases the efficiency of feeding rates are reduced: when infected by common parasites, three-spined stickleback fish, *Gasterosteus aculeatus*, demonstrate a preference for smaller prey resulting in the assimilation of less nutritious items, presumably to reduce competition with uninfected fish (Milinski 1984; Brunner et al. 2017; Anaya-Rojas et al. 2019).

In natural populations, the causal direction between infection and ecological niche shift can be difficult to ascertain. Infection can lead to host niche shifts, but niche-use may alternatively influence a potential host's exposure to certain parasites (Britton &

Andreou 2016). Because exposure to parasite communities can vary greatly across trophic niches, they can be used to identify an individual's foraging strategy and detect changes over time (Britton & Andreou 2016).

Through the use of stable isotopes - a continuous measure of energy flow through trophic levels and communities - it is possible to estimate the trophic niche of individuals. In particular, the ratio of nitrogen stable isotopes ($\delta^{15}N$) of a consumer is normally enriched by 3-4 ‰ in comparison to their prey. Carbon ratios ($\delta^{13}C$) vary much less throughout a trophic web (approximately 1 ‰), and instead provide information on the original source of the carbon, thus revealing the foraging habitat of an organism (Post 2002). In marine ecosystems, a lower $\delta^{13}C$ signature is found in individuals foraging in oceanic foraging areas, while coastal foragers have less depleted $\delta^{13}C$ (Post 2002). With stable isotope analysis, it is therefore possible to explore the link between foraging ecology and parasite burden. For example, infection in fish correlates with both depleted (Britton et al. 2011) and enriched $\delta^{15}N$ values (Welicky et al. 2017), implying a modification of feeding ecology.

The combined effects of infection and feeding ecology may influence resource allocation trade-offs between different life history traits, such as those associated with reproduction and survival (Lochmiller & Deerenberg 2000; Durso & French 2018). In response to infection, reproductive output may be either reduced (Møller 1990; Richner et al. 1993; Eizaguirre et al. 2009) or increased (Sorci et al. 1996; Schwanz 2008; Kalbe et al. 2009; Duffield et al. 2017), broadly depending on whether bet-hedging or terminal investment strategies are adopted. Reduced host fecundity may be a direct consequence of resource exploitation by parasites, but may alternatively be indicative of resource divestment from current reproduction to survival (and future reproduction) until the infection has passed - a bet-hedging strategy (Hurd 2001). For instance, triggering an artificial immune response in the lizard *Ctenophorus fordi* reduces host reproductive

investment as quantified by egg mass, because a trade-off exists between current and future reproductive events (Uller et al. 2006). Alternatively, in situations where recovery is unlikely, strategic terminal investment should instead be favoured, resulting in higher reproductive performance in infected individuals during their final reproductive attempts (Schwanz 2008; Kalbe et al. 2009; Brannelly et al. 2016; Duffield et al. 2017). These strategies might co-exist depending on individuals' life stages. In the blue-footed booby, *Sula nebouxii*, the reproductive success of individuals mounting an immune response is lower in young males, whereas older males nearing senescence show an increase in breeding success as high as 98% (Velando et al. 2006). Finally, responses to infection may span generations via mechanisms known as trans-generational immune priming, which can be advantageous to host progeny if they share the same pathogenic environment as their parents (Marshall & Uller 2007; Kaufmann et al. 2014b; Pigeault et al. 2016; Roth et al. 2018). Alternatively, mechanisms that modify offspring phenotype in a manner that encourages their dispersal may allow the next generation to avoid exposure to a pathogen (Sorci et al. 1994).

Here, we use data from nine years of intensive monitoring of nesting female loggerhead sea turtles, *Caretta caretta*, in Cape Verde. We used stable isotope analysis to investigate the spatiotemporal occurrence of a leech ectoparasite, *Ozobranchus* sp., and relate it to trophic niche and reproductive investment. Little is known about the life-cycle of this leech, although, as all stages of development have been recorded on their host, it is thought they complete their entire life-cycle on turtles (Mcgowin et al. 2011). *Ozobranchus* sp. are the most likely vector of the chelonid herpesvirus ChHV5, which is associated with sea turtle fibropapillomatosis, a potentially fatal neoplastic condition of sea turtle species (Greenblatt et al. 2005; Jones et al. 2016). The role of *Ozobranchus* sp. as a vector of turtle fibropapillomatosis is of high conservation concern, but nothing is currently known about the direct effects of infection by this leech itself on turtle

health, feeding ecology or reproduction. As sea turtle fibropapillomatosis has not yet been reported in loggerhead turtles in Cape Verde, it makes this population ideal to consider the effects of this parasite independently of this co-infection. Composed of several philopatric nesting groups, the Cape Verde population is the 3rd largest loggerhead sea turtle population in the world and the only significant one in the eastern Atlantic, making its conservation not only of local but also of global concern (Marco et al. 2012; Stiebens et al. 2013).

3.3 Methods

3.3.1 Spatiotemporal trends in infection rate

During the months of July to October from 2010 to 2017, nesting female turtles were sampled from nine islands across the Cape Verde archipelago through a long-term citizen science project (Figure 3.1A). After oviposition, turtles were PIT (AVID) and/or metal (Inconel) tagged to allow for identification during subsequent nesting events (Stiebens et al. 2013). Notch-to-notch curved carapace length (CCL) was measured (± 0.1 cm), and the presence or absence of *Ozobranchus* sp. on the cloaca of individuals was recorded. Samples of the leech were collected in order to confirm species identity. A three-millimetre sample of non-keratinised tissue was taken from the front flipper of each turtle for stable isotope analysis. These data were used to quantify general spatiotemporal trends in infection rate across the archipelago ($N_{turtles}$ = 4,386). In 2018 a further 88 turtles were sampled in this manner on the island of Sal, to contribute to studies on the reproductive impact of infection.

3.3.2 DNA extraction and species confirmation.

In order to identify the leech species infecting turtles, we extracted DNA from 90 leeches randomly selected over space and time using the DNeasy 96 Blood and Tissue

kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. We then amplified 654bp of the Nicotinamide adenine dinucleotide dehydrogenase subunit 1, NADH as well as 600bp of the 18S small subunit ribosomal DNA gene. We used primers LND300 (TGGCAGAGTAGTGCATTAGG) and HND1932 (CCTCAGCAAAATCAAATGG, Light & Siddall 1999) for NADH and the modified primers from Medlin et al. (1988) for the 18S rDNA. PCR reactions and thermos-cycling protocols can be found in the supplementary material (Appendix 3, Table A3.1).

3.3.3 Infection and turtle foraging strategy

To determine $\delta^{15}N$ and $\delta^{13}C$ isotope ratios in adult female turtles, tissue samples from a random sub-sample (n = 926) of the population were washed in distilled water to remove contamination from sand, and dried for 48 hours at 60 °C (Cameron et al. 2019). Between 0.7 and 1.3 µg of ground sample were measured into 4 mm tin capsules. A continuous flow isotope ratio mass spectrometer (Integra2, Sercon) combusted the samples, and concurrently analysed both $\delta^{15}N$ and $\delta^{13}C$ elements. Only samples collected from an individual's first recorded nesting event of the season were included in order to maintain an accurate representation of foraging strategy prior to the nesting migration (Cameron et al. 2019).

3.3.4 Infection and reproductive success

The clutches of a subset of females (n = 244) were relocated to *in situ* experimental hatcheries immediately after oviposition. The hatcheries were outdoor enclosures located on the beach that protected nests from tidal inundation and predation, while still allowing them to be exposed to natural environmental conditions. These relocation studies occurred in 2016 and 2017 on the island of Boavista (n = 20 and 39 respectively), and 2017 and 2018 on the island of Sal (n = 97 and 88 respectively). The

number of eggs per clutch was recorded, and for 126 clutches the mass and diameter of two randomly selected eggs were also measured. Clutch mass was calculated as a product of the average mass of these two eggs and the number of eggs in the clutch. Nest success was calculated as the percentage of eggs that resulted in a successfully emerged hatchling.

3.3.5 Trans-generational impacts of infection

The incubation duration of nests was defined as the number of days between oviposition and hatchling emergence. Upon emergence, between 20 and 25 hatchlings from each nest ($N_{nest} = 244$) were randomly selected for fitness trait measurements. Hatchling size is thought to correlate with swimming performance, and so the hatchlings were weighed, and the notch-to-notch straight carapace length (SCL) was measured using digital callipers (± 0.01 mm) (Scott et al. 2014A; Booth & Evans 2011). Two further fitness traits involved in natural predator avoidance during hatchling emergence, crawl speed and time to self-righting, were determined for offspring from 186 of these nests. Crawl speed was measured by recording the time for an individual to crawl the length of a 0.5 m piece of PVC guttering, lined with sand with a dull red light placed at one end. This trial was repeated twice, and an average was taken (cm/s). Self-righting capacity was measured by placing a hatchling on its back on an area of flat sand and recording the time to right itself (Maulany et al. 2012). This trial was repeated three times per individual, and if the hatchling took longer than 60 seconds to self-right it was considered to have failed the trial. We measured both the number of successful trials (0-3) and the average self-righting time (using successful events) in seconds.

3.3.6 Statistical analyses

Statistical analyses were conducted in R version 3.3.3. All models were backwards selected using AIC values to retain the optimal reduced model. Where there were colinearities between fixed variables, we replaced one of these variables with the residuals of its regression against the second. With this approach we could distinguish between variation explained by both predictors in the larger models. A full description of all models can be found in the supplementary material (Appendix 3, Table A3.2).

The spatiotemporal pattern in parasite presence/absence across the archipelago was measured using a binomial generalised linear model (GLM) with year, island and turtle size (CCL) as predictors, along with all two-way interactions. To determine the seasonal trend within the sampling years, a binomial generalised linear mixed effect model (GLMM) that included month, island and their interaction as fixed predictors was used, with year included as a random effect.

Linear mixed effect models (LMM) were used to determine the relationship between infection and feeding ecology. Two separate models, for $\delta^{15}N$ and $\delta^{13}C$ respectively, were conducted with parasite presence/absence, CCL and their interactions as fixed predictors. As differences in feeding strategy have been observed between islands we included island as a random factor, along with year (Cameron et al 2019).

The effect of infection on reproductive parameters (egg mass, egg density, clutch mass and clutch size) was established using independent LMMs that included year and island as random effects, and parasite presence/absence along with $\delta^{15}N$ and $\delta^{13}C$, as well as their interactions as fixed effects. As CCL is a well-known correlate of clutch size and reproductive investment in turtles (Hays & Speakman 1992), it was included as a covariate. Percentage nest success was arcsine transformed before being included in an LMM, again including $\delta^{15}N$, $\delta^{13}C$ and CCL and interactions as fixed effects and with year and islands as random factors. Hatchling fitness was measured as individual size

and mass as well as crawling and righting trials. LMMs for hatchling size and mass included parasite presence/absence, $\delta^{15}N$ and $\delta^{13}C$, CCL, incubation duration and clutch size, and their two-way interactions, whilst crawling and self-righting trials also included hatchling mass.

3.4 Results

3.4.1 Clarifying leech taxonomy.

Out of 90 randomly selected leeches, we successfully retrieved 67 and 86 sequences from18S rDNA and NADH respectively, representing all specimens. All sequences confirmed turtles are infected with the *Ozobranchus margoi* leech – a sea turtle specific sanguivorous parasite (Rodenbusch et al. 2012).

3.4.2 Spatiotemporal trends in infection rate

There was substantial variation in infection rates among islands - infection was lowest in Fogo (FG, 1.33% ± 1.33 SE) and highest in Santiago (ST, 27.95% ± 9 SE). Parasite presence was significantly higher in turtles nesting on the eastern islands of Santiago (ST, 27.95% ± 9.00 SE), Boavista (BV, 19.46% ± 5.47 SE), Maio (MA, 27.15% ± 5.70 SE) and Sal (SAL, 24.69% ± 8.34 SE) than those in the western region of the archipelago (Sao Nicolao, SN: 7.62% ± 7.61 SE, Sao Vicente, SV: 6.18% ± 2.85, Santo Antao, SA: 5.04% ± 2.74 SE, Santa Luzia, SL: 2.13% ± 0.91 SE, Fogo, FG: 1.33% ± 1.33 SE) (Figure 3.1A & B, $X^2 = 145.34$, df = 1, $p < 0.001$). While a significant interaction between island and year suggested different island-specific trends in nesting turtle infection rates over time ($X^2 = 38.357$, df = 8, $p < 0.001$), overall we detected an increase in the prevalence of *O. margoi* in turtles from Cape Verde since 2010 (Figure 3.1C), with yearly oscillations. On average, infected turtles were slightly smaller than uninfected ones (Mean infected: 81.3 ± 0.39 SE cm, Mean uninfected: 82.45 ± 0.19 SE

cm; X^2 = 12.529, df = 1, p < 0.001). We also found evidence of within-year parasite dynamics, with infected turtles being significantly more likely to be encountered at the beginning of the nesting period than later (Figure 3.1D, X^2 = 5.902, df = 1, p = 0.015). Model summaries can be found in Appendix 3, Table A3.3.

3.4.3 Impacts of infection on foraging strategy

There was a strong positive correlation between $\delta^{15}N$ and $\delta^{13}C$ signatures (Appendix 3, Figure A3.1, $F_{1,924}$ = 84.603, p < 0.001) suggesting turtles foraging at a higher trophic level also use more $\delta^{13}C$ enriched areas. Turtle size, measured as CCL and controlled for island ($F_{8,4289}$ = 39.933, p < 0.001), showed a significant positive relationship with $\delta^{15}N$ ($F_{1,826}$ = 10.865, p < 0.001) but not with $\delta^{13}C$ ($F_{1,823}$ = 0.241, p = 0.624). These results vary from previous results from this population that focused on the island of Boavista alone, where both $\delta^{15}N$ and $\delta^{13}C$ correlated with size (Eder et al. 2012). The results from both years, however, are consistent with those of Cameron et al (2019). We found that both the $\delta^{15}N$ and $\delta^{13}C$ were significantly lower in infected than uninfected individuals (Table 3.1, δ15N: Figure 3.2A, $F_{1,828}$ = 9.551, p = 0.002; $\delta^{13}C$: Figure 3.2B, $F_{1,822}$ = 7.562, p = 0.006). This amounted to a reduction of 0.66 ± 0.40 (SE) ‰ for $\delta^{15}N$ and 0.47 ± 0.17 ‰ for $\delta^{13}C$. Overall, infected turtles occupied a slightly modified trophic niche in comparison to uninfected individuals (Figure 3.2C).

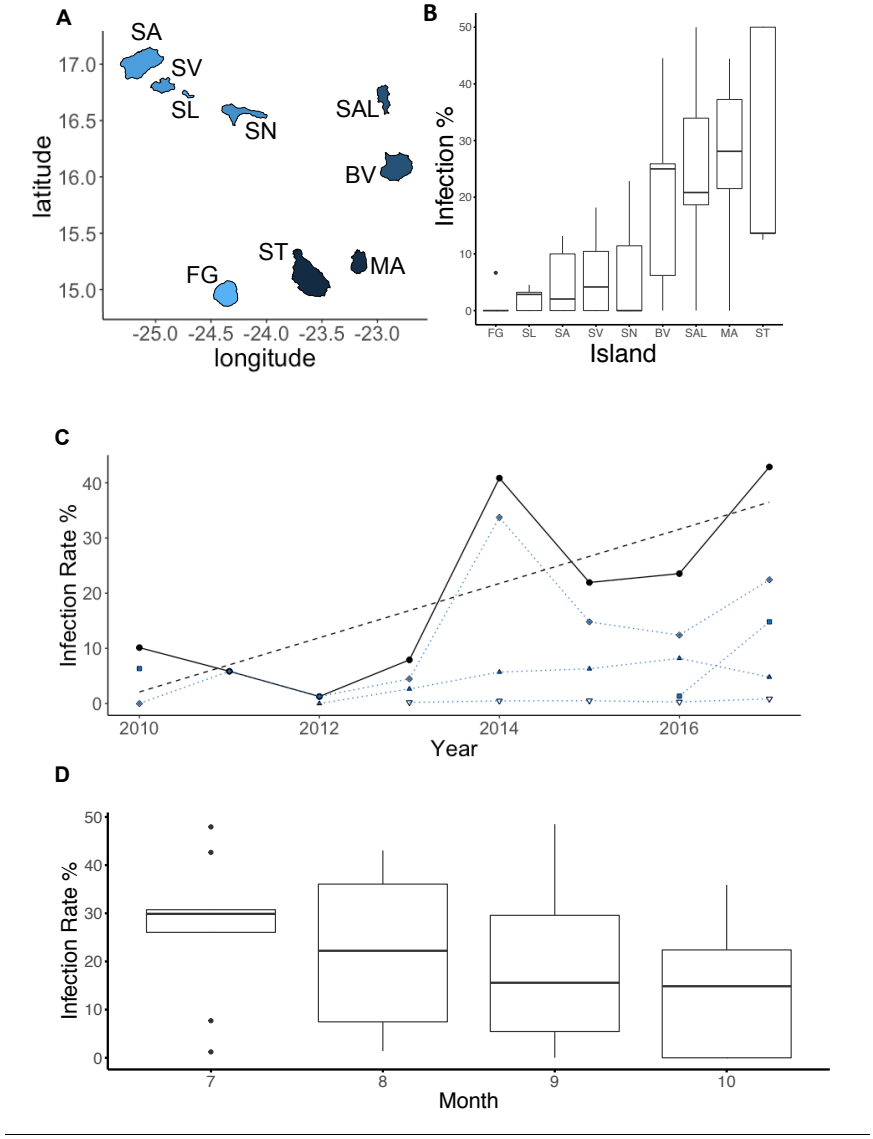

Figure 3.1: Spatiotemporal trends in *O. margoi* infection. A) Map of the islands of Cape Verde, with colour showing average infection rate as corresponding to B; B) Boxplots of infection rate from turtles of nine islands where sampling was conducted. C) Scatterplot shows overall significant in increase infection rate between 2010 and 2017 (black). Only infection rates for the eastern islands are shown because of the longer time series of data available. Diamond = Boavista, solid triangle = Maio, square = Sal and empty triangle = Santiago. D) Mean monthly infection rates across the nesting season.

Table 3.1: Summary table reporting the best reduced models testing the effect of infection and CCL, along with their two-way interaction on 1) $\delta^{15}N$ and 2) $\delta^{13}C$. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

1) $\delta^{15}N$	*d.f.*	F	p
Parasite Presence	**1,828**	**9.551**	**0.002**
CCL	**1,826**	**10.865**	**0.001**
2) $\delta^{13}C$			
Parasite Presence	**1,822**	**7.562**	**0.006**
CCL	1,823	0.241	0.624

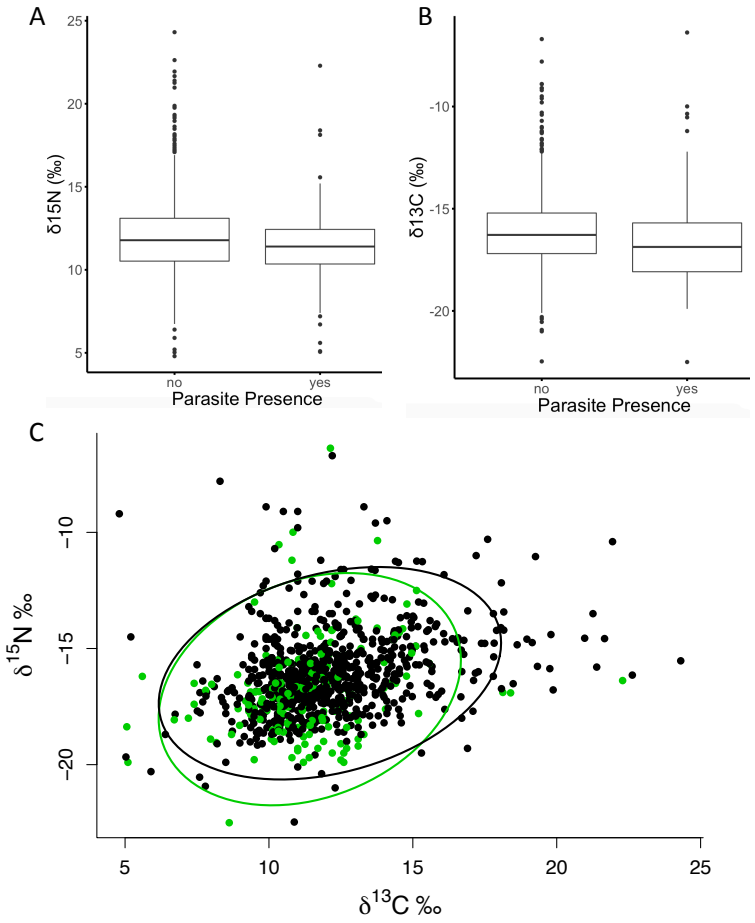

Figure 3.2: Infection is associated with a difference in foraging ecology; A) Mean $\delta^{15}N$ signatures ($F_{1,828} = 9.551$, $p = 0.002$) and B) $\delta^{13}C$ ($F_{1,822} = 7.562$, $p = 0.006$) of infected and uninfected females (with standard error bars). C) Scatterplot showing difference in trophic niche of infected (green) and uninfected (black) individuals.

3.4.4 Impact of infection on reproductive output

Carapace length was the only maternal phenotypic character that correlated positively with the size and mass of individual eggs, with egg size increasing by 4.33 mm ± 0.58 SE with a 10 cm increase in maternal CCL, and egg mass increasing by 1.67 g ± 0.66 SE (Appendix 3, Figure A3.2, size: $F_{1,99} = 37.672$, $p < 0.001$; mass: $F_{1,119} = 54.319$, $p < 0.001$). We also found a significant interaction of maternal CCL and infection on clutch size (Figure 3.3A, $F_{1,128} = 7.400$, $p = 0.007$) and overall clutch mass (Figure 3.3B, $F_{1,110} = 7.802$, $p = 0.006$). Due to the possibility of this relationship being driven by the effect of four very large turtles (> 95 cm), we also ran models excluding these individuals and found that the relationships remained significant (Clutch size: $F_{1,123} = 3.986$, $p = 0.048$, Clutch weight: $F_{1,108} = 5.08$, $p = 0.026$). Specifically, the positive slope describing the relationship between CCL and clutch size varies between infected and non-infected individuals. While non-infected individuals increased their clutch size by 7 ± 2.5 (SE) eggs for every 10 cm increase in carapace length, infected individuals produced 9 ± 3.7 (SE) more eggs per 10 cm carapace length increase. This variation in slope meant that infected females of 90 cm in length produced 15.9% more eggs than their non-infected counterparts, which also resulted in greater clutch mass (Table 3.2). If we apply this measured effect of infection on reproductive output to the clutch size and size structure of turtles nesting on Boavista, then infection would result in a 1.21% net increase in reproductive output.

Although $\delta^{15}N$ and $\delta^{13}C$ were not associated with any characteristics of reproductive investment, $\delta^{15}N$ did show a positive relationship with the success of clutches produced from infected mothers (Appendix 3, Figure A3.3, Table 3.2, $F_{1,126} = 10.731$, $p = 0.001$). An interaction between maternal infection and CCL was also significantly correlated with nest success, whereby there was a positive correlation between CCL and success in uninfected turtles, but not infected turtles ($F_{1,100} = 9.361$, $p = 0.003$).

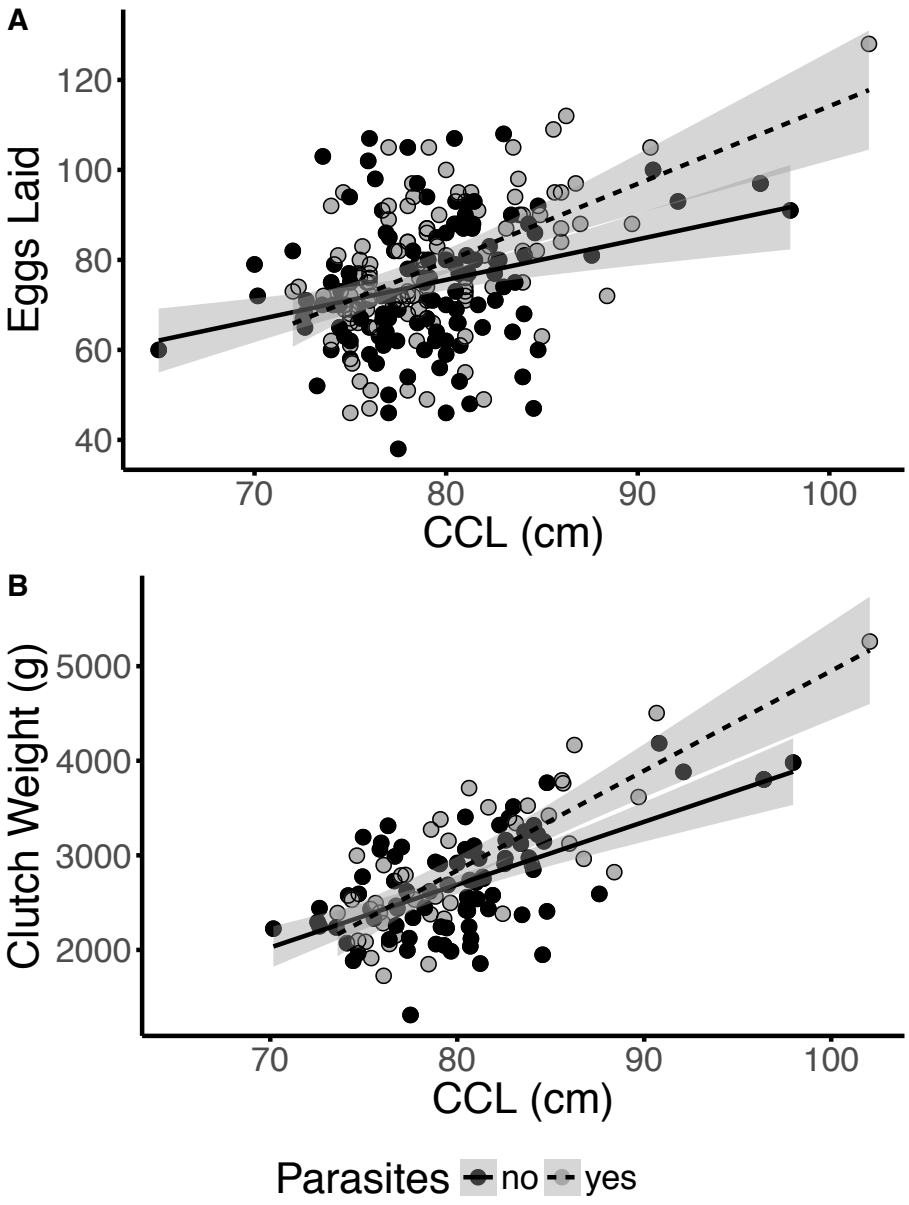

Figure 3.3: Scatterplots show that infection changes the slope of of the relationship between maternal size and both A) clutch size ($F_{1,128}$ = 7.400, p = 0.007) and B) clutch mass ($F_{1,110}$ = 7.802, p = 0.006). Infected females produce larger clutches, particularly so at large size. While plots are bivariate, statistics reported are from final reported multiple regression models.

Table 3.2: Summary table reporting the best reduced models testing the effect of infection, CCL, $\delta^{15}N$ and $\delta^{13}C$, along with their two-way interactions, on reproductive investment, including; 1) Average egg size, 2) Average egg mass, 3) Clutch size, 4) Clutch mass and 5) Nest success. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

1) Average Egg Size	***d.f.***	**F**	**p**
Parasite Presence	1,113	0.38	0.534
CCL	**1,99**	**37.672**	**< 0.001**
$\delta^{13}C$	1,72	0.465	0.497
Parasite Presence: $\delta^{13}C$	1,112	2.027	0.157
2) Average Egg Mass			
CCL	**1,119**	**54.319**	**< 0.001**
3) Clutch Size			
Parasite Presence	1,127.00	3.799	0.053
CCL	1,127	0.589	0.444
$\delta^{15}N$	1,107	0.1	0.752
Parasite Presence:CCL	**1,128**	**7.4**	**0.007**
CCL: $\delta^{15}N$	1,127	2.371	0.126
4) Clutch Mass			
Parasite Presence	1,113	3.873	0.051
CCL	**1,112**	**103.936**	**< 0.001**
$\delta^{15}N$	1,21	2.625	0.119
Parasite Presence:CCL	**1,110**	**7.802**	**0.006**
5) Nest Success			
Parasite Presence	1,125	0.243	0.623
CCL	1,115	0.178	0.674
$\delta^{15}N$	1,122	1.924	0.168
Parasite Presence:CCL	**1,100**	**9.361**	**0.003**
Parasite Presence: $\delta^{15}N$	**1,126**	**10.731**	**0.001**
CCL: $\delta^{15}N$	1,126	3.891	0.051

3.4.5 Transgenerational impact of maternal infection on offspring fitness

An interaction between maternal infection and clutch size was significantly associated with offspring SCL (Appendix 3, Figure A3.4, $F_{1,226} = 6.921$, $p = 0.009$): With incubation duration and maternal size accounted for in the model, hatchling SCL

reduced with clutch size more in non-infected females (reduction of 0.42 ± 0.08 mm/10 egg increase) than in infected females (reduction of 0.11 ± 0.01 mm/10 egg increase). While an interaction between infection and clutch size correlated with hatchling SCL (Table 3.3, $F_{1,226} = 6.921$, $p = 0.009$), this effect did not remain in the reduced model of hatchling mass. The interaction between maternal infection and clutch size also significantly correlated with self-righting speed (Appendix 3, Figure A3.4, $F_{1,114} = 8.413$, $p = 0.004$), in which offspring from infected mothers righted themselves on average 17% faster than offspring from uninfected mothers (average self-righting speed of offspring from infected mothers: 6.51 ± 0.22 (SE) seconds; uninfected mothers: 7.84 ± 0.19 (SE) seconds), but this difference was strongest when clutch sizes were small. The same interaction between maternal infection and clutch size was also detected when investigating with self-righting success ($X^2 = 3.681$, df = 1, $p = 0.055$), but for crawl speed ($F_{1,111} = 2.939$, $p = 0.089$).

Table 3.3: Summary table reporting the best reduced models testing the effect of infection, CCL, $\delta^{15}N$ and $\delta^{13}C$, incubation duration and clutch size along with their two-way interactions, on offspring phenotype including 1) Size and 2) Mass. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

1) Hatchling Size	***d.f.***	**F**	**p**
Parasite Presence	1,225	3.416	0.070
CCL	**1,231**	**4.497**	**0.035**
Incubation Duration	**1,232**	**6.121**	**0.014**
Cutch Size	**1,225**	**19.292**	**< 0.001**
Parasite Presence:Clutch Size	**1,226**	**6.921**	**0.009**
CCL:Incubation Duration	**1,232**	**5.769**	**0.017**
2) Hatchling Mass			
CCL	**1,313**	**29.904**	**< 0.001**
Incubation Duration	**1,208**	**5.955**	**0.020**
Clutch Size	**1,223**	**7.499**	**0.007**

An interaction between hatchling mass and maternal $\delta^{15}N$ value was significantly associated with both self-righting (Appendix 3, Figure A3.5, $F_{1,2525} = 5.163$, $p = 0.023$) and crawl speeds (Appendix 3, Figure A3.5, $F_{1,2458} = 4.993$, $p = 0.026$). Offspring from females with a higher $\delta^{15}N$ value were faster in self-righting tests, but had a slower crawl speed, although this was dependent on the mass of the individual, with this effect being greatest in the heaviest hatchlings. The same interaction was seen between mass and $\delta^{13}C$ on self-righting speed ($F_{1,2526} = 5.163$, $p = 0.023$), with those offspring from a mother with enriched $\delta^{13}C$ performing better in this test, again with the effect being greatest in the heaviest offspring (Appendix 3, Table A3.4).

3.5 Discussion

Long-term monitoring of endangered species enables the observation of population dynamics and the determination of responses to environmental change (e.g. Lachish et al. 2011A). Parasites and pathogens can have especially drastic effects on populations suffering from concurrent anthropogenic pressures, and monitoring their impacts is of prime interest in conservation (e.g. Devil Facial Tumour Disease in Tasmanian devils: Hawkins et al. 2006; white-nose syndrome in bat species: Langwig et al. 2012; chytridiomycosis in amphibians: Scheele et al. 2019). In the current study, we found that over nine years, the prevalence of infection by the sanguivorous leech *Ozobranchus margoi* in Cape Verde has increased, from 10% of the sampled population being infected in 2010, to 33% in 2017. Due to the role of *O. margoi* as a possible vector for sea turtle fibropapillomatosis (FP), this rise could increase the risk of introduction of FP in this rookery. We propose that transmission of *O. margoi* primarily occurs in the feeding ground, based on more depleted $\delta^{13}C$ values of infected turtles, which is associated with oceanic foraging (see Eder et al 2012, Cameron et al 2019 for determination of feeding ground). Although there was little effect of maternal infection

on reproduction for most size classes, the largest infected turtles produced more eggs per clutch with bigger offspring than their uninfected counterparts, while the smallest infected individuals produced fewer eggs and small hatchlings. Offspring from all infected turtles performed better in self-righting tests, which could provide evidence of positive maternal effects associated with infection. Using size as a broad proxy for age, we suggest that the cost of infection could be borne differently across life stages. We propose that while the smallest/youngest infected turtles may be more likely to bet-hedge in favour of lifetime reproductive success, older turtles appear to adopt a terminal investment strategy. Noteworthy, these coexisting strategies result in a slight (1.21%) net increase in the reproductive output of this population compared to a theoretical turtle population without parasites. This could suggest that the evolution of these strategies has managed to ensure that the population avoids the classic costs of parasite infection.

The sanguivorous leech, *O. margoi,* can infect most, if not all, sea turtle species (Davies & Chapman 2011). Since 2010, *O. margoi* prevalence in the Cape Verde loggerhead population has increased, while showing the classic oscillations of parasite infection (Decaestecker et al 2007, Eizaguirre et al 2012). These multiyear oscillations are likely caused by a complex interaction between environmental factors, host demography and host-parasite dynamics, as would be predicted by the Red Queen hypothesis (Van Velan 1973; Greenman et al. 2004; Altizer et al. 2006; Decaestecker et al. 2007). Alternatively, as female turtles nest every two to three years, these oscillations could stem from a cohort effect. However, we consider this explanation less likely because return rates in Cape Verde do not seem to be fixed for a given individual (unpub. Data). The fact that the timing of oscillations is similar across islands implies that infection occurs outside the nesting grounds, rather than within island-specific breeding habitats (Stiebens et al 2013).

Our results also reveal that infection rates are not homogenous across islands, as levels of infection are significantly higher in the east of the archipelago, where nesting densities are higher (Stiebens et al 2013). The existence of island-specific variation in immune genes of the major histocompatibility complex within this sea turtle population could explain differential levels of local adaptation in the host and hence this distribution (Stiebens et al. 2013). Alternatively, it could be that some density-dependent transmission is maintained in large nesting groups, for instance, during mating. Increasing prevalence of this parasite also implies an increased risk from the ChHV5 virus, responsible for FP (Greenblatt et al. 2004). This virus has now been recorded in all species and all ocean basins (Herbst 1994; Greenblatt et al. 2004). If it reaches Cape Verde, the increasing prevalence of its vector may pose a considerable threat to this vulnerable population.

When investigating how parasite infection correlates with feeding ecology, we found a relationship between parasite presence and reduced values of $\delta^{15}N$ and $\delta^{13}C$. Two hypotheses exist to explain these relationships: i) turtles foraging at lower trophic levels are more frequently exposed to and infected by the parasites (e.g. Bertrand et al. 2008 in brook charr, *Salvelinus fontinalis*) or ii) infection reduces foraging efficiency causing turtles to target more prey items from lower trophic positions, which are probably smaller and of lower nutritional value than optimal (Venesky et al. 2009; Naug 2014). Most turtles from Cape Verde forage in oceanic habitats, both opportunistically on neustonic organisms such as jellyfish, and also at higher trophic levels in regions exposed to upwelling events (Frick et al. 2009; Cameron et al. 2019). A much smaller proportion forage in neritic waters providing a different environment and population size of turtles which may not be optimised for the transmission of the parasite (Hawkes et al. 2006, Cameron et al 2019). The lower $\delta^{13}C$ signature seen in infected turtles is indicative of open-ocean foraging, suggesting that transmission of *O. margoi* occurs

most frequently in oceanic feeding grounds (Hatase et al. 2002; Eder et al. 2012; Pikesley et al. 2015; Cameron et al. 2019). If this working hypothesis is correct, it may be that infection of neritic turtles could occur at lower rates in the coastal habitat or during mating. Of note, several studies have recorded the presence of multiple genetically distinct sea turtle populations within the same foraging grounds (Bass et al. 2004; Bjorndal & Bolten 2008; Vásquez-Carrillo et al. 2020). It is possible that this mixing could further elevate the risk of sea turtle FP being transmitted to the Cape Verde population.

From an evolutionary perspective, modified feeding capacity may translate into altered lifetime reproductive success and ultimately impact fitness (e.g. Eizaguirre et al 2009). Infected turtles that foraged at enriched $\delta^{15}N$ levels (associated with nutritious upwelling regions) are on average larger, and produced clutches that had a greater rate of success than those infected individuals with a lower $\delta^{15}N$. High levels of productivity within upwelling regions could allow turtles to forage more efficiently, and compensate from the costs of infection and mounting energetically costly immune responses.

The $\delta^{15}N$ and $\delta^{13}C$ values of individuals did not show a relationship with any characteristics of reproductive investment. Instead, we found that the relationship between maternal size and clutch size was influenced by the presence of parasites. While this relationship may not be detected in all size categories, the smallest infected turtles produced fewer eggs per clutch than their uninfected equivalents. In turn, smaller and lighter offspring hatched from those clutches. This relationship was reversed in the largest infected turtles, which produced bigger clutches and heavier offspring than uninfected conspecifics. As sea turtles grow continuously throughout their life, we may expect that on average, larger sea turtles are older (Omeyer et al. 2017). Hence we suggest these results could point at the coexistence of two size/age specific reproductive

strategies. The smallest, i.e. young, turtles follow a bet-hedging strategy, whereby if infected, they reduce investment, to reserve resources for future reproductive attempts (Bonneaud et al. 2003). On the other hand, large, older, turtles terminally invest, with infected individuals maximising their current reproductive success as there may be few, in any, reproductive events in the future (Agnew et al. 2000). As transition between these two strategies is context-dependent (i.e. parasite infection) and therefore unlikely to be a discrete event, this explains why little effect of infection is detected in sea turtles of moderate size. If we apply the relationship between adult size and clutch size for these two reproductive strategies to the current size class structure in Boavista, we observe a small 1.21% net increase in reproductive output of this population. This is in contrast to the strong negative effect of parasites at the population level that has frequently been observed in bird and mammal populations (Watson 2013). Our results highlight the evolutionary role of host-parasite dynamics, which leads to the evolution of strategies that maximise reproductive success.

Offspring fitness correlated with adult female clutch size, with hatchlings from all infected females performing 17% faster in self-righting tests than offspring from uninfected mothers. Whilst this difference was not mirrored in the crawl tests, it provides some evidence that trans-generational maternal effects confer fitness benefits that may contribute to dispersal (Sorci et al. 1994). Particularly, combined with the negative correlation between self-righting time and offspring mass, maternal effects may maximize dispersal capacity as larger offspring are known to have better swimming capacity than smaller ones (Scott et al. 2014A). An elevated body condition enables offspring to access currents that propel them away from predatory rich coastal areas (Scott et al. 2014A).

Long-term field monitoring projects will provide the first evidence of changing host-parasite dynamics within a population. Such studies are therefore crucial for effective

conservation management. Here, we find that there has been a large increase in the presence of *O. margoi* within sea turtles in Cape Verde since 2010, increasing potential exposure of this population to the often-fatal sea turtle FP. It is possible that *O. margoi* infection itself could also be costly to loggerhead sea turtle hosts, which may have evolved different reproductive strategies based on their size. Interestingly, by combining the effects of infection with the size class structure of the island of Boavista, we observed a net increase in reproductive output of 1.21% in response to *O. margoi* infection, illustrating how monitoring the effects of an infection aids our understanding of population demographics. Future studies will need to determine the cause of the increase in parasite prevalence and determine whether it is host density or environmentally mediated. This study provides the necessary basis for such studies.

5.6 Acknowledgements

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Chapter 4: Hormone-related evolutionary trade-offs in the loggerhead sea turtle *Caretta caretta*

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4.1 Abstract

Finite energy reserves require animals to trade-off resource allocation among multiple physiological and cellular functions, causing the concurrent expression of beneficial and disadvantageous traits. Such trade-offs can be mediated by hormonal pleiotropy – the parallel action of hormones on multiple target pathways. Here, we test whether circulating concentrations of the sex steroid hormones testosterone (T) and oestradiol (E_2) are associated with known life-history trade-offs in loggerhead sea turtles nesting in Cape Verde. We question whether sea surface temperatures, parasite infection or migratory behaviours correlate with individual sex steroid hormone variation, and are subsequently associated with immunity and/or reproduction trade-offs. Current sea surface temperatures did not correlate with any of the measured variables. However, we found a possible E_2-related trade-off between immunity and reproduction which suggests that turtles with high E_2 may elicit an immune response that prevents parasite infection, at the cost of investment into egg mass. We hypothesise that E_2 is could be a proximate mediator of a bet-hedging response to infection previously reported in this population. In hatchlings, we found a T-related trade-off between growth and hatchling vigour, linked to maternal transgenerational investment of testosterone. Overall, we provide evidence for a proximate role of sex steroid hormones as physiological regulators of important trade-offs in a natural population of sea turtles.

4.2 Introduction

Life history theory posits that individuals simultaneously allocate finite energy resources to multiple pathways including growth, immunity and reproduction (Stearns 1989; Sheldon & Verhulst 1996; Speakman 2008). Limited resource availability causes trade-offs between these physiological processes, and the concurrent expression of both beneficial and unfavourable traits (Stearns 1989; Speakman 2008). Hormones regulate gene expression, and thus are involved in in the control of development, physiology, and expression of life history traits (Kempenaers et al. 2008; Cox et al. 2016). Importantly, a single hormone may act upon many pathways simultaneously, i.e. hormonal pleiotropy, mediating proximate mechanisms for many trade-offs (Finch & Rose 1995; Ketterson & Nolan Jr 1999; Williams 2012).

A prominent example of a hormone-mediated trade-off is shown by the sex steroid hormone testosterone (T) in the immunocompetence handicap principle (Hamilton & Zuk 1982; Folstad & Karter 1992). T is involved in the expression of sexual behaviours and secondary sexual traits such as aggression and ornamentation in breeding males (Marler & Moore 1988; Milinski & Bakker 1990; Verhulst et al. 1999). The allocation of T to these traits is associated with the concurrent impairment of cell-mediated immunity and the increased susceptibility to parasite infection (McKay & Cidlowski 1999; Roberts et al. 2004; Cornelius et al. 2014; Foo et al. 2016). This trade-off facilitates female choice, as only males of good genetic quality can maintain the concentrations of T necessary for elaborate ornamentation (Folstad & Karter 1992; Eizaguirre et al. 2009; Foo et al. 2016).

T also mediates trade-offs between growth and activity, as experimentally demonstrated with eastern and northern fence lizards (*Sceloporus undulatus* and *S. undulatus hyacinthus* respectively), whereby elevated T in the months prior to mating correlated negatively with growth rates in male lizards, but positively with activity levels and the

size of home ranges (Klukowski et al. 1998; Cox et al. 2005; John-Alder et al. 2009). Similar patterns of growth vs. behaviour are observed in juvenile birds, as T reduces growth rates but increases territorial activity in black headed gull chicks *Larus ridibundus* (Ros 1999).

While male sex steroid hormones are well studied, there are still knowledge gaps when it comes to females (Tannenbaum et al. 2019). The primary sex hormone involved in female reproduction is oestradiol (E_2). E_2 initiates vitellogenesis, which is the production of yolk pre-cursor proteins, vitellogenins, in the liver (Ho et al. 1982). This process is energetically costly and is associated with both a 30% increase in metabolism of snakes, and the mobilisation of body fat reserves in many vertebrates (Hamann et al. 2002; Dyke & Beaupre 2011; Price 2017). On the other hand, E_2 also impairs cell-mediated immunity, but has an immunoenhancing effect on humeral responses (Klein 2004; Foo et al. 2016). As humeral immune responses are less costly than cell-mediated immune responses, a pleiotropic effect of E_2 on humeral responses might allow females to direct resources towards reproduction while maintaining immune function (Lee 2006; Foo et al. 2016).

The neuroendocrine and endocrine systems facilitate the link between environmental perception and functional response (Wingfield 2008). As anthropogenic activities drive global climate change, we can expect the disruption of hormonal cascades restructuring existing trade-offs (Meylan et al. 2012). For example, climate warming reduces concentrations of E_2, and consequently reproductive output, in cinnamon anamonefish *Amphiprion melanopus* (Miller et al. 2015). The recent colonisation of a warmer environment by the dark-eyed junco *Junco hyemalis* led to an extended breeding season and prolonged periods of T elevation, but lower peak concentrations (Atwell et al. 2014). Identifying how hormone-mediated trade-offs may be impacted by environmental change may provide early warning signals to changes in population

dynamics that might threaten wild populations (Clements et al. 2017; Baruah et al. 2019).

As long-lived ectotherms, marine turtles are highly dependent on their thermal environment. Trade-offs between immune function and reproduction appear to exist in nesting females, whereby parasites infection leads to reduced reproductive investment in the smallest turtles (bet-hedging), but the largest turtles rather follow a terminal investment strategy (Chapter 3). While the evolution of such strategies is now well understood, the proximate mechanisms remain elusive and may stem from hormone-mediated trade-offs. Importantly for sea turtles, T and E_2 influence the temperature-dependent sex determination mechanism (Chapter 2). Understanding how these two hormones co-vary within a multifaceted environment will help elucidate their effects on sex determination in the face of climate change (Witt et al. 2010; Laloë et al. 2014). For instance, circulating testosterone concentrations in free-swimming juvenile sea turtles positively correlate with eight-day averages of sea surface temperatures (SST), suggesting that a physiological response to a warming environment exists (Hawkes et al. 2013).

Here, we test how circulating T and E_2 in nesting female loggerhead turtles (*Caretta caretta*) correlate with environmental conditions during the nesting season. We define the environment by SST (abiotic), parasite infection (biotic) and migration (behaviour). We include migration because stress during this life history stage causes both reduced and increased testosterone concentrations in migratory birds, which are linked to carry-over effects on reproductive success (Bauchinger et al. 2009; Crossin et al. 2012). In the loggerhead turtle population of Cape Verde, different feeding strategies exist which are associated with different migration routes, as confirmed by satellite tracking and stable isotope analysis (Hawkes et al. 2006; Eder et al. 2012; Pikesley et al. 2015; Cameron et al. 2019). The majority of turtles undertake migrations to oceanic waters approximately

400 km from their nesting grounds where they forage across a wide home range, and are more exposed to parasite infection (Chapter 3). At the same time, a subpopulation migrates three times further to coastal waters near Sierra Leone, where they feed in a small area of the continental shelf (Hawkes et al. 2006). This difference provides a natural experiment by which we can compare the correlations of different migration strategies with hormone concentrations. Furthermore, by coupling variation in T and E_2 with reproductive output, we test whether the two hormones correlate with proximate mechanisms that mediate trade-offs between the environment and reproductive fitness. Lastly, we quantify variation in circulating hormone concentrations in hatchlings, to test whether hormone concentrations have a heritable basis, and to test for the existence of growth-fitness trade-offs during this crucial early life history stage.

4.3 Methods

4.3.1 Adult sampling

Data was collected on the island of Boavista, Cape Verde, between the 25th July and 21st September 2016 (n = 134 $_{turtles}$), and between 17th July and 1st August 2017 (n = 28 $_{turtles}$). Of the turtles sampled in 2016, eight individuals were sampled twice and five individuals three times. The sampling site is a 400 m beach on the southern tip of the island (15°58'18.6"N, 22°48'06.2"W). Immediately after oviposition, between 1 and 4 ml of blood was taken from the dorsal cervical sinus using a 40 mm, 21-gauge needle and 5 ml syringe, and stored in lithium heparin coated tubes. Turtles were individually PIT (AVID) and/or metal tagged (Inconel), and 3 mm samples of non-keratinised tissue were taken from the front flipper for stable isotope analysis (Stiebens et al. 2013, Cameron et al. 2019). We recorded whether each turtle was infected with the leech parasite *Ozobranchus margoi* (Species verified in Chapter 3), and measured the turtles' curved carapace length (CCL, ± 0.1 cm) and width (CCW, ± 0.1 cm).

4.3.2 Reproductive output and offspring fitness

To investigate correlations between hormone variation and reproductive output, we relocated nests to an experimental field hatchery. This is an enclosed area on the same beach as where the nest was deposited, protected from predators and tidal inundation, but otherwise exposed to natural environmental conditions. Twenty nests were relocated in 2016, and a further 28 in 2017. We placed a TinyTag™ temperature logger at the centre of each clutch, programmed to collect readings every 15 minutes for the entire duration of incubation. The numbers of eggs within a clutch were counted (clutch size), and two eggs from each clutch were measured with digital callipers (± 0.1 mm) and weighed using a digital scale (± 0.1 g). Clutch mass was calculated by multiplying the average egg mass by clutch size. Success rate was determined as the percentage of hatchlings that successfully developed and emerged alive from the nest.

Upon emergence of hatchlings from 28 of the 2017 nests, 20 individuals were randomly selected, weighed using a digital scale (± 0.1 g), and their notch-to-notch straight carapace length (SCL) was recorded (± 0.1 mm) using digital callipers. We conducted two different tests linked to offspring dispersal. Firstly, crawl tests measured the speed of individuals: we recorded the time an individual took to crawl 50 cm of a runway, with a dull red light at the end to attract the hatchling. This trial was repeated twice, and an average was calculated (cm/s). Secondly, we conducted self-righting tests, in which a hatchling was placed on its back on flat sand and the righting duration was recorded. This was repeated three times. Each trial was considered a success if righting was achieved within 60 seconds. The average of successful trials per individual was used for statistical analyses. Finally, blood samples (100 – 150 µl) were taken from the dorsal cervical sinus, using a 26-gauge needle and 1 ml syringe (Wibbels et al. 1998), and stored within lithium heparin coated tubes. All blood samples from both adults and

hatchlings were refrigerated for up to 48 hours before being centrifuged to separate the plasma, which was then frozen at -20 °C until hormone extraction.

4.3.3 Hormone extraction

T and E_2 were extracted from plasma samples using commercial kit protocols (E_2: Catalogue # ADI-900-174, T: Catalogue # ADI-900-065, ENZO Life Sciences). Anhydrous diethyl ether was added to plasma samples of volume 40 - 200 µl at a ratio of 5:1. After homogenising, samples were flash frozen and the diethyl ether fraction was decanted into a fresh tube. Depending on starting sample volume, either 0.5 or 1 ml of distilled water was added, and the solution was homogenised once more. The subsequent organic fraction was removed and evaporated over two hours using a speed vacuum. Samples were then rehydrated using 250 µl of appropriate assay buffer from the ELISA kits, and frozen until assayed.

Serial dilutions of known hormone concentration were prepared according to kit protocol to produce standard curves (E_2: n = 7, 1000 – 15.6 pg/ml; T: n = 5, 2000 – 7.81 pg/ml). All samples were run in duplicate using a Fluostar Omega plate reader (BMG Labtech), with concentrations calculated using the MARS program. Extraction efficiencies were calculated for both hormones by dividing samples (E_2 = 6, T = 5) into two aliquots, and spiking one with a known concentration of E_2 (272 pg/ml) or T (400 pg/ml) prior to the extraction process. Extraction efficiency was calculated as 54.5 ± 10.5 (SE) % for E_2, and 43.9 ± 2.8 (SE) % for T. The average inter-assay coefficient of variation (CV) for E_2 was 14.53% and the intra-assay CV was 11.39%. The inter-assay CV for T was 9.52%, and the intra-assay CV was 10.72%.

Due to insufficient starting quantities of plasma, hormones were not extracted from all turtles. We successfully extracted E_2 from the plasma of 98 of the 134 turtles in 2016 and all (n = 28) turtles in 2017, and extracted T from 73 of the 134 individuals in 2016

and 23 of the 28 turtles in 2017. Of these turtles, both hormones were extracted for 69 turtles in 2016, and 22 in 2017. We successfully extracted hormones from a mean of 13 ± 4 (SD) individuals from each nest. Hatchling sex was estimated as per the method reported in Chapter 2.

4.3.4 Stable isotope analysis

The $\delta^{15}N$ and $\delta^{13}C$ isotope signatures of skin samples were used to categorise the foraging strategy of individuals. $\delta^{15}N$ informs on the trophic position of an individual, which is on average enriched by 3-4 ‰ in comparison to their prey. A higher $\delta^{15}N$ signature therefore indicates a higher trophic level, or feeding in an environment with an elevated $\delta^{15}N$ baseline, such as upwelling areas (Cameron et al. 2019). $\delta^{13}C$ ratios vary of approximately 1 ‰ between trophic positions, and provide an indication of the carbon source, thus informing about habitat type use (Post 2002). A depleted $\delta^{13}C$ signature indicates an oceanic foraging strategy, while coastal foragers have a less depleted $\delta^{13}C$ signature (Post 2002). We washed skin samples in distilled water to remove sand, and dried these for 48 hours at 60 °C. Samples were ground, and 0.7 – 1.3 µg were measured into 4 mm tin capsules (Cameron et al 2019). We combusted the samples using a continuous flow isotope ratio mass spectrometer (Integra2, Sercon), which analysed both elements simultaneously. Foraging strategy was defined using affinity propagation clustering within R (version 3.3.3), using the *apcluster* package. This approach considers the similarity of each data point to others through machine learning to identify clusters of similar points, and importantly, does not require the number of clusters to be determined a priori (Frey & Dueck 2007), or to define specific thresholds which may vary as a function of environmental fluctuations (Cameron et al. 2019). These clusters were then converted into foraging strategies by comparing their $\delta^{13}C$ and $\delta^{15}N$ with the Cape Verde population foraging strategies described in Cameron

et al. (2019). In 2016, if turtles had been sampled more than once we used their first $\delta^{15}N$ and $\delta^{13}C$ values, as these should be most representative of their foraging strategy, as self-assimilation or possible feeding at the breeding ground could bias isotope ratios later in the season (Cameron et al. 2019).

4.3.5 Statistical analyses

All analyses were conducted using R version 3.3.3. Colinearities of independent variables were removed using the residuals of their relationship, with both variables then used within the main models. Models were then backwards selected using AIC criteria to retain the most reduced model. Linear models (LM) were used to compare $\delta^{15}N$ and $\delta^{13}C$ values between foraging strategies and years. A linear mixed effects model (LMM) was used to correlate T and E_2 in the circulating plasma of adults, using year as a random factor. We correlated T with E_2 in hatchlings using an LM, and included sex as an additional predictor, along with its interaction with E_2. The differences between median circulating T and E_2 in adults and hatchlings, and also between adults and hatchlings, were assessed using the Wilcoxon rank sum tests because of the skewed distributions of the hormones.

To test temporal variation in hormone concentrations, we used data from adult nesting turtles in 2016. As turtles return to their nesting grounds at different times, we considered each sample in relation to an individual's first recorded nesting event (days from first recorded nesting). This represented a change in hormone concentrations linked to physiological nesting requirements across the season. Using LMs with E_2, T and the E_2:T ratio as response variables, we included days from first recorded nesting event, and an average of SST in the two weeks prior to this (downloaded from the NOAA National Centres for Environmental Information) as predictors. If turtles were sampled more than once across the nesting season, we included only the first sample

within analyses to avoid pseudo-replication. We used a two-week SST average to test for short-term temperature effects on physiology. Alongside these variables, we also included CCL, infection status (parasite presence/absence), either foraging strategy or raw $\delta^{15}N$ and $\delta^{13}C$ values, and the two-way interactions of all variables. Using the smaller 2017 dataset, we used LMs to test whether similar relationships occurred in the following year. Due to the smaller sample size and lack of temporal variation between samples, in these models we included CCL, infection and foraging strategy/raw $\delta^{15}N$ and $\delta^{13}C$ values and their two-way interactions as predictors.

Reproductive investment was quantified in terms of egg size and mass, the number of eggs laid (clutch size), the overall mass of the clutch (clutch mass), and the percentage of eggs within a nest that hatched successfully (success rate). LMs were used to test whether adult T and E_2 correlated with these variables, by including both hormones as fixed effects, along with female $\delta^{13}C$ signature, infection status, CCL, year and all two-way interactions. Success rate was analysed with a binomial generalised linear model (GLM) to fit model requirements of percentage data.

Using LMMs that included nest ID as a random factor, we tested for relationships between hatchling hormone concentrations (E_2, T and E_2:T as response variables) and both individual phenotype (body mass index, BMI, which we calculated as a function of individual mass/size) and the clutch incubation environment (mean incubation temperature). In these models we also included maternal hormone concentration, $\delta^{13}C$ ratio and infection status, to ascertain any trans-generational effects on offspring fitness since those variables were associated with fitness-correlated traits in the maternal dataset. We did not include hatchling sex in these models, as we were specifically interested in the effects of E_2 and T individually. Finally, we investigated the effect of hatchling circulating hormone E_2 and T on both crawl and self-righting speeds. In two

LMMs that used nest ID as a random effect, we included BMI, clutch size, $\delta^{13}C$ and infection status, and all two-way interactions as fixed effects.

4.4 Results

4.4.1 Defining foraging strategy by isotopic cluster

Overall, $\delta^{15}N$ and $\delta^{13}C$ values showed a significant positive correlation ($F_{1,157} = 10.301$, $p = 0.002$). Individual $\delta^{13}C$ values differed between years ($F_{1,157} = 55.484$, $p < 0.001$), but this was not mirrored in $\delta^{15}N$ values ($F_{1,157} = 2.177$, $p = 0.142$). As $\delta^{13}C$ changed between years, we defined separate annual isotopic clusters for 2016 and 2017 (Appendix 4, Table A4.1, Figure A4.1). In 2016, affinity propagation clustering found that turtles were grouped into one of three foraging strategies, matching those found previously in this population (Cameron et al. 2019). Two of these showed depleted $\delta^{13}C$ values, characteristic of an oceanic feeding strategy (mean ± SD, Group 1: -15.68 ± 1.07 ‰, Group 2: -17.16 ± 0.84 ‰). These groups were separated by a significant difference in $\delta^{15}N$ values ($F_{1,70} = 96.612$, $p < 0.001$, mean ± SD Group 1: 14.22 ± 0.96 ‰, Group 2: 11.75 ± 0.91 ‰), indicative of one group foraging at a higher trophic level, that is associated with upwelling areas. The third group showed significantly higher $\delta^{13}C$ values which may be linked to a neritic foraging strategy ($F_{2,89} = 55.680$, $p < 0.001$, mean ± SD $\delta^{13}C$: -14.71 ± 1.05 ‰, $\delta^{15}N$: 11.72 ± 0.62 ‰, Eder et al. 2012, Cameron et al. 2019). Two foraging strategies were detected in 2017, and these also demonstrated a clear distinction between neritic (mean ± SD $\delta^{13}C$: -11.33 ± 1.99 ‰, $\delta^{15}N$: 13.56 ± 2.21 ‰) and oceanic (mean ± SD $\delta^{13}C$: -15.37 ± 0.59 ‰, $\delta^{15}N$: 12.39 ± 1.17 ‰) strategies.

4.4.2 Individual variation in circulating hormone concentrations

Distribution of circulating concentrations of E_2, T and the E_2:T ratios in the plasma of

both nesting females and hatchlings were positively skewed (Appendix 4, Figure A4.2) - a common characteristic of hormone data (Pollet & van der Meij 2017). In adults, variation was an order of magnitude greater in T than in E_2 (T: median: 1088.86 pg/ml, interquartile range (IQR): 193.57 – 2414.22 pg/ml, E_2: median: 159.56, IQR: 65.25 – 286.25; Wilcoxon ranksum = 9413, $p < 0.001$). Hormone concentrations were significantly lower in hatchlings (T: Wilcoxon ranksum = 30233, $p < 0.001$, E: Wilcoxon ranksum = 32272, $p < 0.001$), with E_2 being significantly higher than T (Wilcoxon ranksum = 32071, $p < 0.001$, T median: 49.08 pg/ml, IQR: 32.59 – 71.58 pg/ml; E_2 median: 82.19 pg/ml, IQR: 59.16 – 106.89 pg/ml). Concentrations of E_2 and T were positively correlated in both adults (Appendix 4, Figure A4.3A, $F_{1,91} = 74.909$, $p < 0.001$). This was matched in hatchlings, where the slope of the correlation was greater for female offspring (Appendix 4, Figure A4.3B, $F_{1,357} = 11.824$, $p < 0.001$).

4.4.3 Variation in the circulating hormone concentrations of adult females

Investigating how hormone concentrations varied over time, we found that T concentrations significantly decreased by 36.76 ± 16.01 (SE) pg/ml per day across the nesting period, relative to an individual's first recorded nesting event (Figure 4.1A: $F_{1,32} = 4.478$, $p = 0.042$). Days from first nesting was not a statistically significant predictor for E_2 concentrations ($F_{1,53} = 2.529$, $p = 0.118$), but the E_2:T ratio increased by 0.03 ± 0.01 (SE) per day as the nesting season progressed (Figure 4.1B: $F_{1,33} = 4.286$, $p = 0.046$).

Testing whether hormone concentrations varied with environmental factors, here, the average SST two weeks prior to nesting, we did not find any correlation for either hormone individually, but the E_2:T ratio of individuals was reduced when SST was higher ($F_{1,33} = 8.386$, $p = 0.007$, Table 4.1).

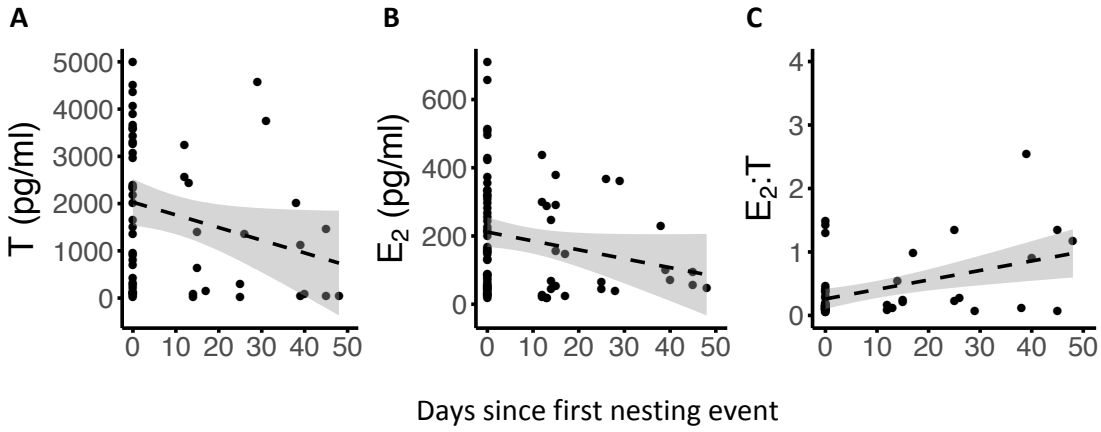

Figure 4.1: Scatterplots showing that in the plasma of nesting females A) there was a significant decrease in circulating T over the nesting period ($F_{1,32}$ = 4.478, p = 0.042); B) this relationship was not significant for E_2 ($F_{1,53}$ = 2.529, p = 0.118); C) there was an increase in the E_2:T ratio ($F_{1,33}$ = 4.286, p = 0.046). While plots are bivariate, statistics reported are from final reported multiple regression models.

Foraging strategy as identified by affinity propagation clustering did not correlate with either hormone or the E_2:T ratio (Table 4.1). However, when we considered $\delta^{13}C$ and $\delta^{15}N$ independently, we found a significant interaction between $\delta^{13}C$ and parasite infection in relation to T (Figure 4.2A: $F_{1,32}$ = 8.408, p = 0.007). Specifically, T in non-infected turtles increased by by 73.12 ± 34.45 pg/ml with every 1‰ of $\delta^{13}C$, yet this relationship was not seen in infected individuals. We found a positive relationship between E_2 and $\delta^{13}C$, with E_2 increasing, on average, by 55.72 ± 17.44 (SE) pg/ml with every 1‰ of $\delta^{13}C$ in all individuals ($F_{1,53}$ = 6.675, p = 0.013). Similarly, there was a significant interaction between $\delta^{15}N$ and parasite infection on E_2 concentrations, whereby no correlation existed in uninfected turtles, but E_2 in infected turtles was low, and decreased by 86.22 ± 50.35 pg/ml for an increase of 1‰ $\delta^{15}N$ (Figure 4.2B: $F_{1,53}$ = 4.687, p = 0.035). These significant findings were not detected in the smaller, short-term 2017 dataset (Appendix 4, Table A4.2). Together, these results suggest that

infected turtles mostly use oceanic feeding grounds, and that trade offs appear upon infection.

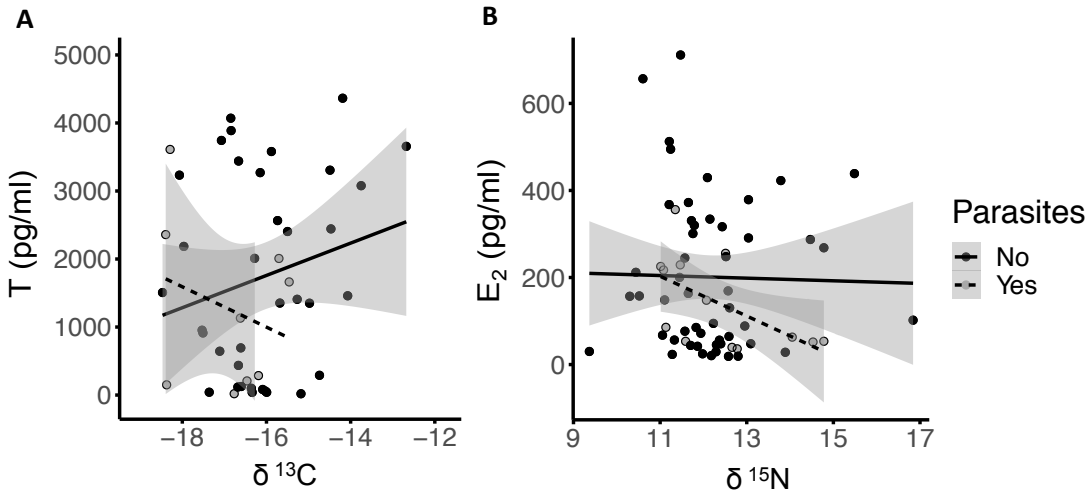

Figure 4.2: Scatterplots showing A) T correlated with an interaction between $\delta^{13}C$ and infection ($F_{1,32} = 8.408$, $p = 0.007$) B) E_2 correlated with an interaction of $\delta^{15}N$ and infection ($F_{1,53} = 4.687$, $p = 0.035$). While plots are bivariate, statistics reported are from final reduced multiple regression models reported within text.

Table 4.1: Summary table of the best reduced linear models testing the relationships between various environmental/individual variables and E_2, T and the E_2:T ratio for 2016 samples.

Foraging Strategy				**$\delta^{15}N$ and $\delta^{13}C$ as independent variables**			
	d.f	F	p		d.f	F	p
T							
Days	**1,35**	**4.641**	**0.038**	**Days**	**1,32**	**4.478**	**0.042**
SST	1,35	0.141	0.709	SST	1,32	0.196	0.661
CCL	1,35	0.172	0.681	CCL	1,32	0.333	0.568
Strategy	1,35	0.450	0.507	$\delta^{15}N$	1,32	0.172	0.681
Parasites	1,35	1.410	0.243	$\delta^{13}C$	1,32	2.100	0.157
Days:SST	1,35	1.629	0.210	Parasites	1,32	0.482	0.492
SST:CCL	1,35	0.698	0.409	Days:Parasites	1,32	1.419	0.242
CCL:Strategy	1,35	2.353	0.134	SST:CCL	1,32	0.137	0.713
				SST:Parasites	1,32	0.065	0.800
				CCL: $\delta^{15}N$	1,32	2.439	0.128
				$\delta^{15}N$: $\delta^{13}C$	1,32	0.641	0.429
				$\delta^{15}N$:Parasites	**1,32**	**3.251**	**0.081**
				$\delta^{13}C$:Parasites	**1,32**	**8.408**	**0.007**
E_2							
Days	1,73	2.399	0.126	Days	1,53	2.529	0.118
				SST	1,53	0.447	0.507
				CCL	1,53	1.410	0.240
				$\delta^{15}N$	1,53	1.031	0.315
				$\delta^{13}C$	**1,53**	**6.675**	**0.013**
				Parasites	1,53	0.788	0.379
				Days:SST	1,53	1.408	0.241
				CCL:Parasites	1,53	1.764	0.190
				$\delta^{15}N$:Parasites	**1,53**	**4.687**	**0.035**
				$\delta^{13}C$:Parasites	1,53	2.186	0.145
E_2:T							
Days	**1,30**	**9.245**	**0.005**	**Days**	**1,33**	**4.286**	**0.046**
SST	**1,30**	**7.089**	**0.012**	**SST**	**1,33**	**8.386**	**0.007**
CCL	1,30	0.099	0.755	CCL	1,33	0.007	0.934
Strategy	1,30	0.025	0.875	$\delta^{15}N$	1,33	2.295	0.139
Parasites	1,30	0.906	0.349	Parasites	1,33	0.207	0.652
Days:SST	1,30	0.194	0.662	Days:CCL	1,33	2.503	0.123
Days:Parasites	1,30	0.733	0.399	SST:CCL	1,33	1.329	0.257
SST:CCL	1,30	0.674	0.418	CCL: $\delta^{15}N$	1,33	3.787	0.060
SST:Parasites	1,30	0.992	0.327	CCL:Parasites	1,33	2.014	0.165
CCL:Parasites	1,30	0.767	0.388	**$\delta^{15}N$:Parasites**	**1,33**	**15.274**	**<0.001**
Strategy:Parasites	**1,30**	**6.344**	**0.017**				

4.4.4 Correlating adult hormone concentrations with reproductive investment

Maternal CCL showed a positive relationship with total clutch size ($F_{1,11}$ = 6.270, p = 0.029), clutch mass ($F_{1,9}$ = 39.826, p < 0.001) and the average size of eggs in a clutch ($F_{1,15}$ = 26.225, p < 0.001). Concentrations of adult hormones, however, did not correlate with these three indicators of reproductive investment (Appendix 4, Table A4.3). While the average egg mass in a clutch was also positively related with CCL (Table 4.2: $F_{1,13}$ = 22.643, p < 0.001), eggs became lighter as adult circulating E_2 concentrations increased (Figure 4.3: $F_{1,13}$ = 7.425, p = 0.017).

Table 4.2: Best reduced linear model correlating adult hormone concentrations, infection status and foraging strategy with the average mass of eggs. As predictor variables we included circulating T and E_2 concentrations in adults, along with $\delta^{13}C$, infection status, CCL, year and all two-way interactions. Significant results in bold

	d.f	F	p
T	1,13	0747	0.403
E_2	**1,13**	**7.425**	**0.017**
CCL	**1,13**	**22.643**	**< 0.001**
$\delta^{13}C$	1,13	0.997	0.336
Year	1,13	1.364	0.264
Parasites	1,13	1.046	0.325
T:CCL	1,13	0.468	0.506
T: $\delta^{13}C$	1,13	0.668	0.429
T:Parasites	1,13	0.040	0.844
E_2:CCL	1,13	0.120	0.734
CCL:Parasites	1,13	0.327	0.577
$\delta^{13}C$:Year	1,13	0.546	0.473
$\delta^{13}C$:Parasites	1,13	3.522	0.083

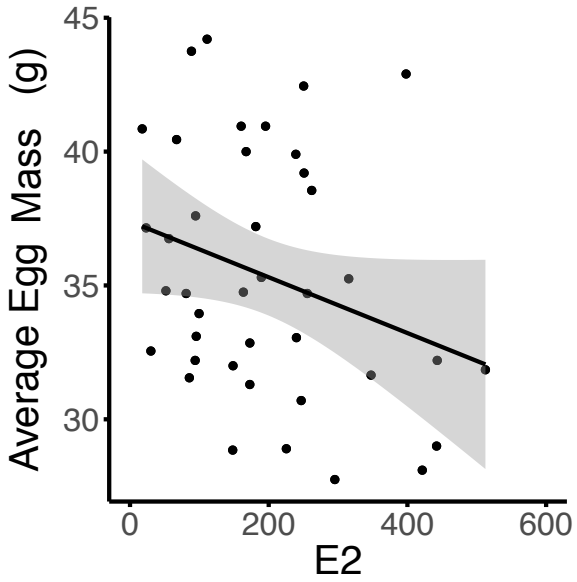

Figure 4.3: Scatterplot showing a negative correlation exists between female E_2 concentrations, and the average mass of eggs ($F_{1,13} = 7.425$, p = 0.017). While plot is bivariate, statistics reported are from final reported multiple regression model.

4.4.5 Hormone variation in hatchlings

In 2017, no characteristics of individuals or maternal phenotypes were significantly linked to hatchling E_2 concentrations (Table 4.3), but concentrations of this hormone did show a positive relationship with incubation temperature (Figure 4.4A: $F_{1,15} = 12.885$, p = 0.003). This relationship did not exist with hatchling turtles' T concentrations (Table 4.3). Instead, hatchling T was associated with an interaction between hatchling BMI and maternal T concentrations, whereby there was a negative correlation between hatchling T and BMI in offspring from mothers with low T (Figure 4.4B: $F_{1,66} = 4.284$, p = 0.042). Finally, hatchling E_2:T concentrations correlated with an interaction of maternal E_2:T ratio and incubation temperature. In hatchlings originating from mothers with a high E_2:T ratio, hatchling E_2:T correlated positively with incubation temperature. In hatchlings originating from mothers with a low E_2:T ratio on the other hand, hatchling E_2:T had a negative relationship with incubation temperature (Table 4.3: $F_{1,7} = 6.268$, p = 0.041).

Table 4.3: Summary of the best reduced models describing the relationships between individual hatchling phenotype, environmental variables and E_2, T and the E_2:T ratio. We included BMI (mass/SCL) of individual offspring and mean nest incubation temperature, along with maternal hormone concentrations, infection status and $\delta^{13}C$ value as fixed effects, along with all two-way interactions. Nest was included as a random effect. Significant results in bold

	E2				T				Ratio		
Response	**df**	**F**	**p**	**Response**	**df**	**F**	**p**	**Response**	**df**	**F**	**p**
BMI	1,76	0.001	0.99	BMI	1,215	0.114	0.735	BMI	1,88	1.103	0.297
Adult E_2	1,15	0.128	0.725	Adult T	1,12	1.593	0.23	**Adult E_2:T**	**1,7**	**7.151**	**0.035**
$\delta^{13}C$	1,18	2.615	0.123	$\delta^{13}C$	1,11	0.001	0.993	$\delta^{13}C$	1,9	2.174	0.175
Parasites	1,16	0.110	0.744	Parasites	1,10	3.230	0.103	**Temperature**	**1,8**	**7.345**	**0.028**
Temperature	**1,15**	**12.885**	**0.003**	Temperature	1,10	0.859	0.376	BMI: $\delta^{13}C$	1,65	2.236	0.139
BMI:Parasites	1,88	3.783	0.055	**BMI:Adult T**	**1,66**	**4.284**	**0.042**	BMI:Temperature	1,31	0.099	0.754
Adult E_2:Parasites	1,15	1.531	0.235	BMI: $\delta^{13}C$	1,217	0.905	0.342	**Adult E_2:T: $\delta^{13}C$**	**1,7**	**6.748**	**0.037**
$\delta^{13}C$:Parasites	1,19	2.785	0.112	BMI:Parasites	1,56	1.466	0.231	**Adult E_2:T:Temperature**	**1,7**	**6.268**	**0.041**
				BMI:Temperature	1,87	1.034	0.312	$\delta^{13}C$:Temperature	1,8	4.632	0.065
				AdT:Temperature	1,10	0.610	0.452				
				$\delta^{13}C$:Parasites	1,10	0.802	0.392				
				$\delta^{13}C$:Temperature	1,8	1.023	0.341				

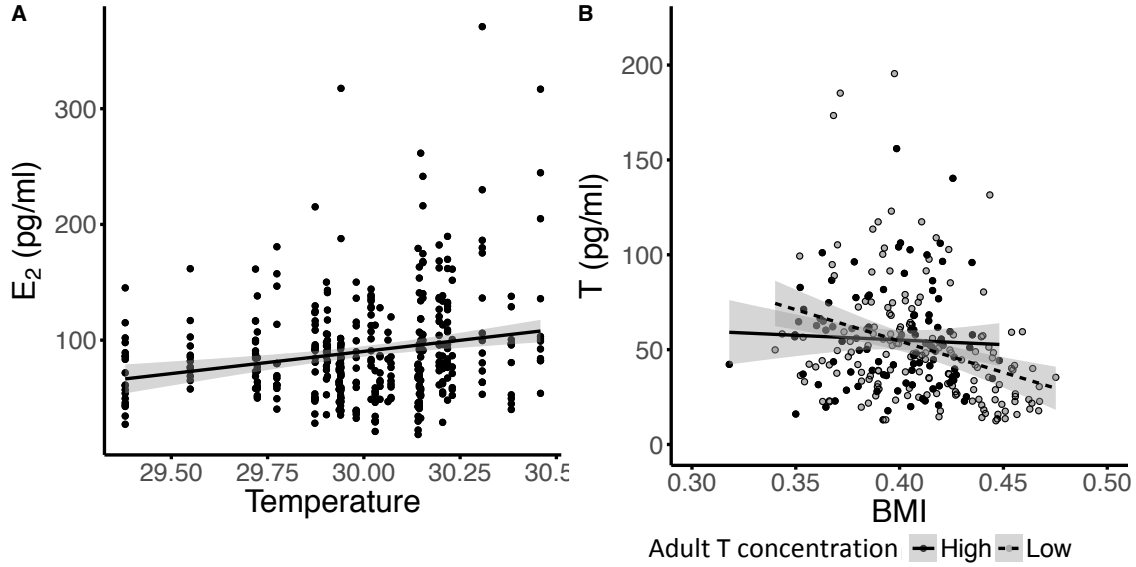

Figure 4.4: Scatterplots showing A) E_2 in hatchlings positively correlated with incubation temperature ($F_{1,15}$ = 12.885, p = 0.003) B) There was a significant interaction between the effect of maternal T and individual BMI on hatchling circulatory T ($F_{1,66}$ = 4.284, p = 0.042). While plots are bivariate, statistics reported are from final reported multiple regression models.

4.4.6 Consequences for hatchling fitness

None of the variables included in our model predicted the self-righting time of hatchlings (Appendix 4, Table A4.4). Individuals with elevated T concentrations were faster in crawl tests, however, when they originated from non-infected mothers compared to those from infected females (Table 4.4, Figure 4.5A: $F_{1,300}$ = 5.962, p = 0.015). There was also an association between crawl speed and the interaction between hatchling E_2 concentrations and whether their mother was infected (Table 4.4, Figure 4.5B: $F_{1,309}$ = 7.464, p = 0.007). However, in this case, individuals with high circulating E_2 concentrations were slower in crawl tests, and this relationship was strongest in offspring from healthy females. These relationships suggest that circulating concentrations of T and E_2 influence initial dispersal from the nest. In order to evaluate

the possible impact of individuals with the most extreme T and E_2 values, fourteen datapoints were removed as possible outliers (T: n = 7, E_2: n = 7, total = 14 individuals). It resulted in the detection of similar patterns, trends and effects of hormones and maternal infection on hatchling crawl speeds (T*Infection: $F_{1,259} = 3.219$, p = 0.073, E_2*Infection: $F_{1,277} = 4.443$, p = 0.036). As a result of these consistent patterns, the full dataset is presented.

Table 4.4: Reduced model describing the relationship between hatchling phenotype and crawl speed. We included hatchling BMI and individual circulating T and E_2 concentrations as fixed effects with their two-way interactions as fixed effects, and also included incubation temperature and maternal strategy and infection status. Nest ID was included as a random effect. Significant results in bold

	d.f.	F	p
T	1,253	0.422	0.516
E_2	1,272	0.001	0.986
BMI	1,288	0.865	0.353
Temperature	1,23	0.005	0.944
Parasites	1,21	1.171	0.291
$\delta^{13}C$	1,22	0.016	0.900
T:Parasites	**1,300**	**5.962**	**0.015**
T:$\delta^{13}C$	**1,212**	**4.441**	**0.036**
E_2Parasites	**1,309**	**7.464**	**0.007**
E_2:$\delta^{13}C$	1,251	3.464	0.064
BMI:Temperature	1,308	1.918	0.167

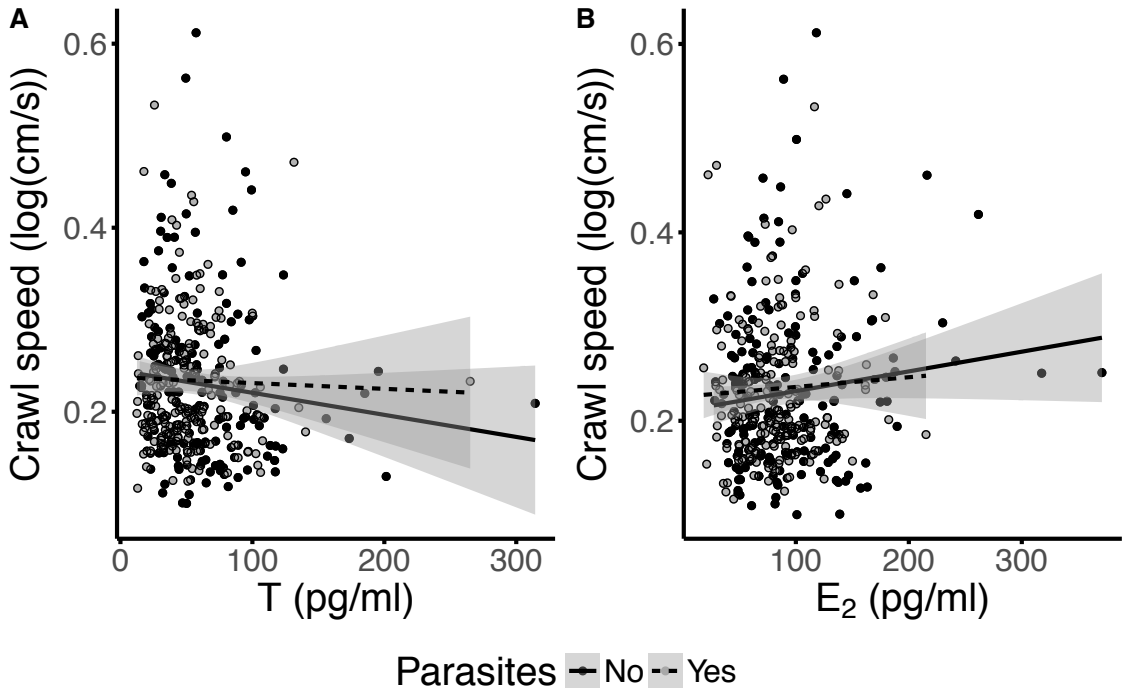

Figure 4.5: Scatterplots showing the significant interactions of parasite infection and A) T ($F_{1,300}$ = 5.962, p = 0.015) and B) E_2 ($F_{1,309}$ = 7.464, p = 0.007) on crawl speed. While plots are bivariate, statistics reported are from final reported multiple regression models.

4.5 Discussion

Exploring endocrine mechanisms in wild populations can help identify the functional links that mediate evolutionary trade-offs (Sheldon & Verhulst 1996). Here, we investigated whether circulating T and E_2 correlate with trade-offs at two life history stages of loggerhead sea turtles. In our 2016 seasonal dataset, circulating concentrations of E_2 showed a positive correlation with the $\delta^{13}C$ isotope value of all nesting female turtles, with this relationship also seen in the T concentrations of turtles that were not infected by leech parasites. As $\delta^{13}C$ is an indicator of feeding region, these relationships could be linked to the varying physiological demands between migration and feeding strategies. We also found possible evidence of an E_2-related trade-off between immune function and reproductive investment, whereby turtles with high E_2 are less likely to be

infected with the leech parasite *O. margoi*, but also invest less energy in reproduction, producing lighter eggs. In hatchlings, a T-related trade-off between growth and locomotor performance appears to occur, particularly in offspring from mothers who have low T concentrations themselves. In this group, individuals with elevated T have faster crawl speeds, which are linked to dispersal ability, but with a cost in terms of reduced growth.

High levels of individual variation occur in both circulating E_2 and T in female loggerhead sea turtles nesting in Cape Verde. If heritable, as some of our results suggest, this could mean the existence of considerable polymorphism in hormone regulation. The positive skew of distributions matches results from other sea turtle populations and many other taxa (Hawkes et al. 2013; Pollet & van der Meij 2017). Recorded concentrations of T and E_2 are extremely different between sea turtle populations worldwide - those reported here are much lower than loggerhead turtles in Florida for example (Myre et al. 2016, Chapter 2, Appendix 2, Table A2.1) This could be the result of local adaptation driven by different physiological requirements to environmental conditions experienced within their feeding or nesting grounds (Monzón-Argüello et al. 2010; Stiebens et al. 2013).

T concentrations decreased significantly across the nesting period, with a concurrent increase in the E_2:T ratio. This is likely the consequence of the physiological regulation of migration and mating behaviour prior to the nesting season (Rostal et al. 1998; Wibbels et al. 1990). Similar decreases in T have been observed in many studies with female loggerhead (Wibbels et al. 1990; Myre et al. 2016), leatherback *Dermochelys coriacea* (Rostal et al. 1996; Rostal et al. 2001), green *Chelonia mydas* (Hamann et al. 2002) and Kemp's ridley *Lepidochelys kempii* turtles (Rostal et al. 1998). This decrease in maternal T and increase in E_2:T might have indirect consequences if circulating hormones predict those invested into egg yolks. Positive correlations between

circulating T in maternal plasma and yolk follicles exist in red-eared slider turtles, and were found also, yet in a non-linear manner, in this population (Chapter 2, Janzen et al. 2002). If the changes in maternal T and E_2:T across the nesting period lead to differential hormone investment in consecutive clutches, there may be a consequence for developing embryos. For example, experimentally elevated yolk testosterone improved offspring body condition in the dragon lizard *Ctenophorus fordi* (Uller et al. 2007), and low concentrations of maternally derived hormones in egg yolks were associated with increased male offspring production at 30 °C in this population (Chapter 2).

Circulating E_2 increased with $\delta^{13}C$ signature while T correlated with an interaction between $\delta^{13}C$ and infection. In this interaction, T correlated positively with $\delta^{13}C$ in non-infected turtles, and negatively in infected individuals, who mostly had low T and low $\delta^{13}C$ values. Overall, the positive relationships generally observed between hormone concentrations with $\delta^{13}C$ suggest that turtles with the highest hormone concentrations use increasingly more coastal waters as foraging areas. Because isotope values are determined from skin samples which have a slow turn over, they reflect the habitats used by turtles during the foraging stage, even months after migration (Eder et al. 2012; Cameron et al. 2019). The vast majority of turtles with a coastal, neritic feeding strategy in this population forage in a small area along the continental shelf near Sierra Leone, with little daily displacement during the foraging period (Hawkes et al. 2006; Pikesley et al. 2015). The migration distance for turtles tracked to this region however, is on average 1253 km. This is a much greater journey than that of oceanic turtles, which displace on average 415 km away from Cape Verde, but travel much further daily during the foraging period (Hawkes et al. 2006). Noteworthy, it is known that some turtles of this population can shift from one feeding strategy to the other as determined by stable isotope analysis of the scutes (Cardona et al. 2017). Furthermore, in this

population, the more oceanic turtles are the most exposed to the leech parasite *O. margoi*, with up to 30% of them being infected (Chapter 3). In sea turtles, hypotheses posit that T instigates a pre-migratory increase in shoulder and pectoral muscle mass, which facilitates swimming during long migrations (Jessop et al. 2004). We theorise that this pre-migratory increase in swimming muscle could be more necessary for relatively stationary coastal turtles with longer migrations, in comparison to oceanic turtles that may maintain elevated muscle mass throughout their life to enable foraging across a much wider home range. Such a mechanism could explain why non-infected, more coastal turtles with longer migrations have higher circulating T within this population. While pre-migratory T increases could be unnecessary in sea turtles foraging in mostly oceanic regions, due elevated exposure to infection these individuals could be required to trade off T with mounting an immune response, explaining our observed interaction. Trade-trade offs involving T are common in the animal kingdom upon infection (Ezenwa et al. 2012; Foo et al. 2016). The most parsimonious explanation for the observed elevation in E_2 is a result of the natural positive correlation between E_2 and T. T is the precursor androgen of E_2, and so it is logical that an increase in T with $\delta^{13}C$ would be mirrored in E_2 (Strauss & FitzGerald 2018).

Sea turtles with the highest E_2 concentrations also show low $\delta^{15}N$ signatures, which are associated with a high proportion of oceanic foraging away from upwelling areas (Cameron et al. 2019), and thus elevated parasite exposure. In these individuals, we found infection was associated with lower E_2 concentrations, which showed a negative relationship with $\delta^{15}N$. Turtles with high circulating E_2 were more likely to mount an immune response, but, importantly, individuals with high E_2 produced lighter eggs This relationship between E_2, infection status and egg mass suggests a potential immunity-reproduction trade-off related to hormone regulation. In Chapter 3, we demonstrated the possible coexistence of reproductive strategies with the smallest infected turtles

following a bet-hedging strategy, while the largest infected turtles likely follow a strategy of terminal investment. The E_2 mediated trade off here might form the proximate mechanism of the bet-hedging response. A recent meta-analysis focusing on sex steroids and immune functions demonstrated that E_2 had a medium-to-large effect on reducing parasite load in experimental studies (Foo et al. 2016). For instance, low E_2 concentrations have been recorded in pygmy rattlesnakes infected with snake fungal disease (Lind et al. 2019). Yet, at the same time this hormone has a crucial role in the costly reproductive process of vitellogenesis, the production of yolk-precursor proteins (Ho et al. 1982; Dyke & Beaupre 2011). This relationship seems to also exist in other reptile species - E_2 has also been negatively correlated with the number of developing follicles in Galápogos marine iguanas (Neuman-Lee & French 2017).

The relationship between hatchling T and the interaction of maternal T and hatchling BMI suggests a degree of heritability of testosterone concentrations, and this was also seen in the E_2:T ratio. Hatchlings had high T concentrations and E_2:T ratios when they originated from mothers with high T and E_2:T ratios respectively. Our findings agree with those in male garter snakes *Thamnophis sirtalis,* where estimates of T heritability between full siblings were near one (King et al. 2004). The heritability of E_2:T ratios is could be mediated by the environment, as this relationship was particularly strong at warm incubation temperatures. If heritability is a function of the environment, it may allow individuals to match their phenotype to the environment, maintaining a sufficiently high adaptive potential to cope with climate change (Chapter 2, Janzen et al. 2002).

Hatchling T was linked to faster performance in crawl tests overall. In individuals originating from mothers with low T concentrations, where there was a low likelihood of inheriting high T, a growth-dispersal T related trade-off was observed, where T was highest in small individuals. T has also been linked to higher concentrations of

endurance and movement, but reduced growth rates in both eastern (*Sceloporus undulatus*) and northern (*S. undulatus hyacinthinus*) fence lizards (Klukowski et al. 1998; John-Alder et al. 2009). Overall these findings suggest there may be a heritable benefit to variation in T, and if offspring inherit low T concentrations, a growth-dispersal trade-off is likely to occur.

We have shown here that circulating concentrations of E_2 and T provide a potential functional link for trade-offs in both adult and hatchling sea turtles. However, as global change progresses, physiological demands of sub-optimal environments may require either of these hormones to be up- or down-regulated (Meylan et al. 2012). Hormonal pleiotropy may consequently lead to alterations of these trade-offs between both infection and reproduction in adults, and growth and dispersal in hatchlings. While it is impossible to predict whether the consequences of such disruption will be beneficial or detrimental, this study highlights the necessity for a deeper understanding of the functional mechanisms that facilitate current trade-offs within a changing world.

4.6 Acknowledgements

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Conclusion

Sea turtles have many traits that make them vulnerable to climate change, and rising temperatures will impact them at all stages of their life-cycle (e.g. Hawkes et al. 2009; Witt et al. 2010; Pikesley et al. 2015). Reproduction and development are, however, two particularly critical stages (Hawkes et al. 2009; Witt et al. 2010). In this thesis, I examined how traits associated with sea turtle breeding biology interact with, and respond to, environmental factors related to global change. To cover new scientific ground, I focused particularly on the under-studied physiological mechanisms that might ultimately maintain population demographics and persistence.

The action of rising temperatures on temperature-dependent sex determination (TSD) will result in increasingly female-biased sea turtle sex ratios over the remainder of this century (Hawkes et al. 2007; Witt et al. 2010; Laloë et al. 2014; Tanner et al. 2019). Strong natal philopatry limits gene flow, and can result in local adaptation of traits, even if turtles only a short time in their nesting areas (e.g. Weber et al. 2012; Stiebens et al. 2013). Surprisingly, the adaptive potential and plasticity of the TSD thermal response curve has never been quantified for sea turtles in relation to their local environment. Instead, models that predict future population demographics mostly use a fixed, non-evolving response (e.g. Laloë et al. 2014).

To date, the gap in understanding TSD in natural populations of sea turtles is mostly associated with the lack of non-lethal methods to determine sex of hatchlings. This constraint limits direct descriptions of thermal response curves. Furthermore, repeated sampling from a single location across multiple years is almost non-existent, and studies are limited to very small sample sizes. Nevertheless, without knowledge of adaptive potential and possible plastic response mechanisms, it is impossible to make accurate predictions of how climate change may affect population demographics (Eizaguirre & Baltazar-Soares 2014; Beever et al. 2016).

In this thesis, my colleagues and I circumvented the further sacrifice of hatchlings to study the TSD mechanism with two very different approaches. First, in Chapter 1, we combined all known records of quantified, population-specific, sea turtle thermal response curves worldwide. Using a space-for-time approach, I demonstrated that over a third of the variation in populations' pivotal temperatures is explained by local temperature and rainfall conditions. This finding provides evidence of local phenotype-environment matching of the sea turtle TSD thermal response curve.

Secondly, in Chapter 2, I developed a non-lethal approach for determining the sex of individual hatchlings, building on previous use of plasma hormone profiles, and combining these with incubation durations – a known proxy for sex in turtles – using machine learning algorithms (Gross et al. 1995; Xia et al. 2011; Mrosovsky et al. 1999). This facilitated the study of how mechanisms, other than temperature, may influence offspring sex determination in unprecedented detail. The finding that maternally-derived yolk hormones may explain variation in offspring sex ratios under standardised thermal conditions is consistent with the role of hormone transfer in other more conventional model TSD species (Bowden et al. 2000; Carter et al. 2017). This study provides experimental evidence of specific physiological plasticity in sea turtles' TSD mechanism, which might allow offspring sex ratios to naturally adjust in response to environmental change.

Taking the combined results of Chapters 1 and 2, I argue that the thermal response curves of sea turtle populations are far more variable than has generally been recognised, and that turtle lineages have evolved mechanisms that allowed them to survive major climate events. I anticipate this conclusion will have implications for population demographics in the future. These findings also fundamentally underline the necessity to consider plastic and adaptive responses to environmental temperature

change and beyond (Eizaguirre & Baltazar-Soares 2014; Beever et al. 2016). Failure to do so could result in poor predictions and the possible failure of management plans.

Despite the hopeful message that sea turtles might have some ability to respond to environmental change, the outlook remains poor. After we updated sex ratio predictions across the century to account for theoretical plastic and adaptive responses, the models presented in Chapter 1 still show that climate change will feminise most populations. Specifically we predict that at least three-quarters of sea turtle populations worldwide could produce over 90% female offspring under realistic warming scenarios that exceed 2 °C above pre-industrial conditions. An important avenue of further research will be to build on our understanding of what such consequences of feminisation will be for the viability of future populations.

Building on this new knowledge, there are several urgent priorities. Most importantly, we need an updated global assessment of sex ratios worldwide. The models developed in Chapter 1 will allow us to estimate a more accurate location-specific T_{piv} and TRT than previously possible. Facilitating the use of these models by conservation organisations will directly inform management plans. Collaborating with conservation organisations will also allow the collection of data that will enable better parameterisation of these models, and exploit this non-lethal method to its full potential. Furthermore, the non-lethal approach to sexing individual hatchlings presented in Chapter 2 can contribute to this approach. With enough resources, we should be able to significantly improve our understanding of current offspring sex ratios, and adjust mitigation plans accordingly.

We should also fine-tune our knowledge of the role of maternal hormones on offspring sex ratios. Are the levels of sex steroid hormones transferred within the yolk heritable? If so, maintaining genetic diversity in populations will be especially important (Stiebens et al. 2013; Eizaguirre & Baltazar-Soares 2014). Are the effects of hormones constant

across temperature gradients, or instead after certain temperature thresholds, are these effects amplified or lost? An experimental approach to answer this question might be to incubate eggs from the same clutch at different, controlled, depths (and therefore different thermal regimes). Such a split-clutch design will standardise maternal egg provisioning while varying temperature. In this way we can examine within-clutch thermal responses, and quantify the relative contributions of temperature and hormone transfer to sex ratios. Understanding these relationships will have downstream implications for conservation decisions. Many conservation organisations relocate eggs to hatchery areas to avoid inundation, predation and the impacts of tourism (Ditmer & Stapleton 2012; Wood et al. 2014). An improved awareness of appropriate temperature regimes will avoid inadvertently deleterious effects on sex ratios, such as the over-production of a single sex, or exceeding lethal temperatures.

The impacts of climate change are not restricted only to abiotic environmental changes. There will also be disruption to species' interactions within communities, as they face trade-offs in an environment that is becoming less well matched to their phenotypes (Harrington et al. 1999). Host-parasite dynamics are key examples of interactions predicted to be impacted by changing environments (Brooks & Hoberg 2007; Brunner & Eizaguirre 2016). In this thesis, I examined how sea turtle infection with the leech *Ozobranchus margoi* changes over time, and how it impacts their reproductive performance. This leech parasite is of conservation importance, being the most likely vector for sea turtle fibropapillomavirus, which can be fatal (Greenblatt et al. 2005; Jones et al. 2016). However, very little was known about the effect of this leech parasite on populations, despite the vast general literature outlining the evolutionary effects of parasites on their hosts (McCallum & Dobson 1995; De Castro & Bolker 2005).

In Chapter 3, I showed that the prevalence of this leech increases over time within the Cape Verde loggerhead turtle population, and that infection is possibly linked to life-

history trade-offs. In this work, I discovered size/age-dependent reproductive strategies in the form of bet-hedging and terminal investment. Specifically, infection in the smallest turtles in the Cape Verde population was associated with individuals investing less energy in current reproductive events, whereas the largest infected turtles invested more energy into reproduction than their uninfected counterparts. Together, the evolution of these reproductive strategies result in the positive growth of the population compared to responses independent of parasite infection. Going forward, continued monitoring of host-parasite dynamics in this system will i) allow us to identify if the parasite begins to negatively impact population growth, which could be an early warning signal of environmental change impacting host-parasite interactions and ii) provide an early detection system for the arrival of fibropapillomavirus in this population.

In Chapter 4, I demonstrated that the immunity-reproduction trade-off associated with parasite infection correlates with circulating plasma concentrations of the sex steroid hormone oestradiol. Hormone-mediated trade-offs may be at risk in the future due to environmental perturbations disrupting upstream hormone cascades (Meylan et al. 2012). Alternatively, they may be vulnerable to artificial chemical endocrine disruptors, which are becoming more common in the environment (Porte et al. 2006). The observed link between oestradiol and both immunity and reproduction in Chapter 4 suggests that artificial oestrogens that inhibit oestrogen receptors may, in particular, be a potential threat to reproductive physiology in sea turtles. At the long-term field sites, it would be pertinent to collect information on water quality and other environmental variables.

Overall, this thesis demonstrates the importance of long-term monitoring of wild populations for truly understanding subtle changes in population demographics. Early warning signals can often be identified years in advance of a population crash, through the detection of increased perturbations in the stability of indicator traits, such as body

size or cohort sex ratios (Clements et al. 2017; Baruah et al. 2019). Such long-term data very rarely exist for wild populations, but will be an invaluable tool for effective conservation. Informal reports of increases in total nesting events at various sea turtle rookeries make a good example here. While one may initially assume this is an indication of population recovery - the result of successful conservation efforts - this may not be the case. If operational sex ratios have already become female-biased with temperature increases for a while, a rapid increase in nesting females might precede a population crash.

For many species on the planet today, their continued existence relies on i) the extent and rate of environmental change (Visser 2008; Bellard et al. 2012); ii) their ability to respond plastically to this change (Chevin et al. 2010; Merilä & Hendry 2014); iii) their adaptive potential (Hoffmann & Sgrò 2011; Eizaguirre & Baltazar-Soares 2014) and iv) in many cases, the willingness of societies to help preserve them. The aims of this thesis were to investigate some of the physiological mechanisms associated with plastic and adaptive responses to the environment, and use this information to explore the consequences of climate change on a vulnerable taxon. Although I have focussed on a single, vulnerable species, understanding the mechanisms that drive species responses to their environment will help to better understand ecological systems. Underpinning conservation with knowledge such as that of this thesis might increase our likelihood of management success, and the maintenance of biodiversity into the future.

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Appendix 1: Supplementary Material from Chapter 1

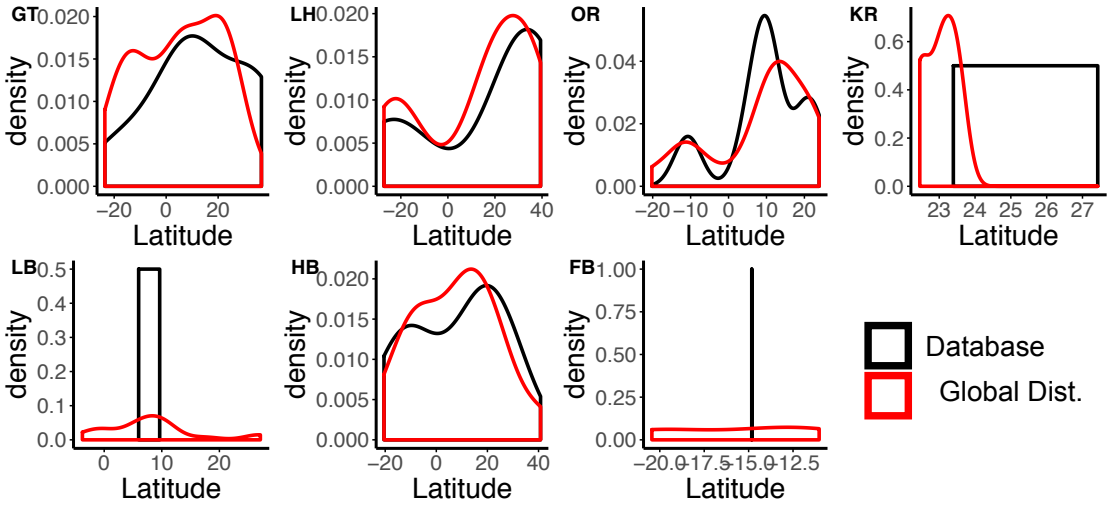

Figure A1.1: Density plots showing the global distribution of sea turtle species, extracted from seaturtle.org, compared to the distribution of populations used within analysis, specifically green (GT), loggerhead (LH), olive ridley (OR), Kemp's ridley (KR), leatherback (LB), hawksbill (HB) and flatback (FB) sea turtles.

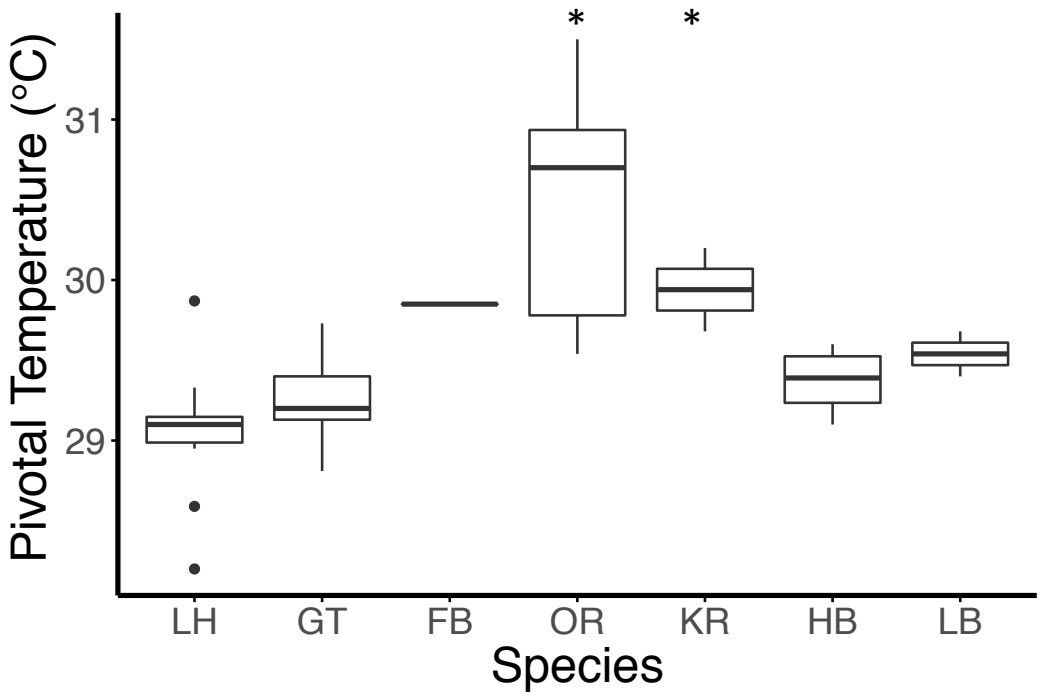

Figure A1.2: Boxplot showing a significant difference between the T_{piv} of species ($F_{6,30}$ = 8.246, $p < 0.001$).

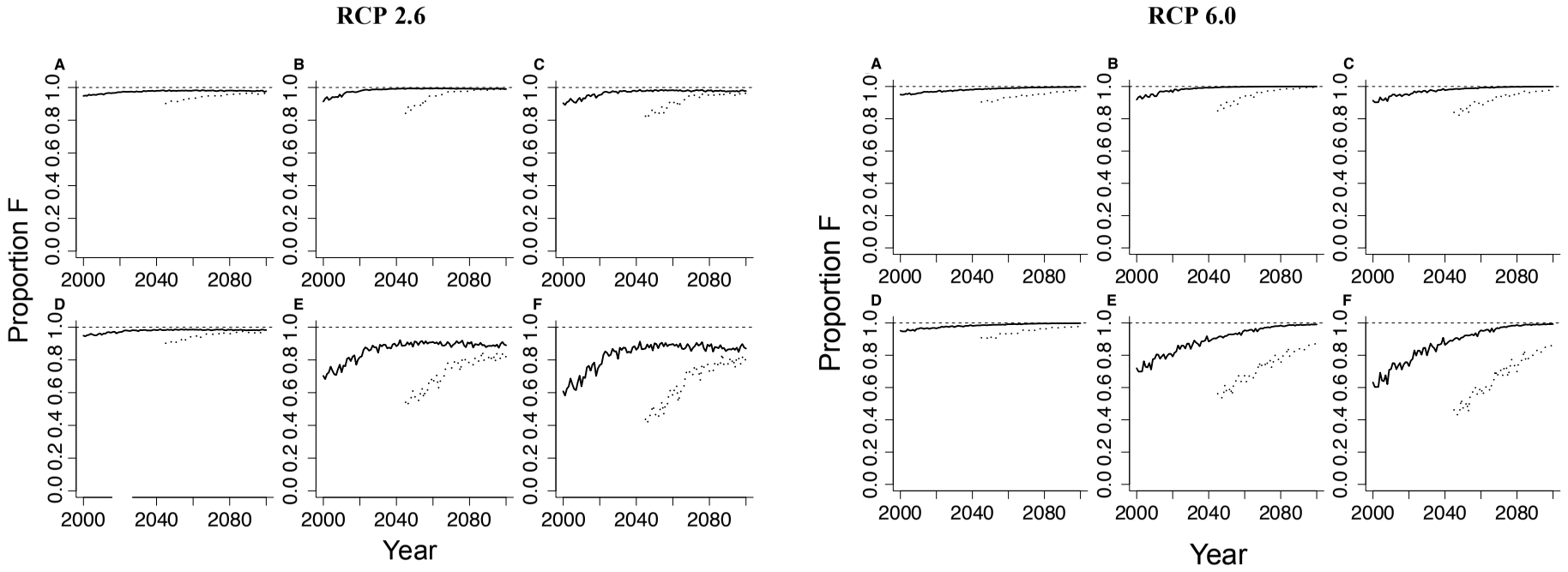

Figure A1.3(i) Time series showing proportion of female hatchlings produced at rookeries in North America and the Caribbean under RCP 2.6 and RCP 6.0 using original reported T_{piv} for sea turtles in A) Texas (LeBlanc et al. 2012) B) Puerto Rico (Mrosovsky et al. 2009), C) Florida (Mrosovsky 1988), D & E) Georgia (LeBlanc et al. 2012; Mrosovsky 1988) and F) North Carolina (Mrosovsky 1988). Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity

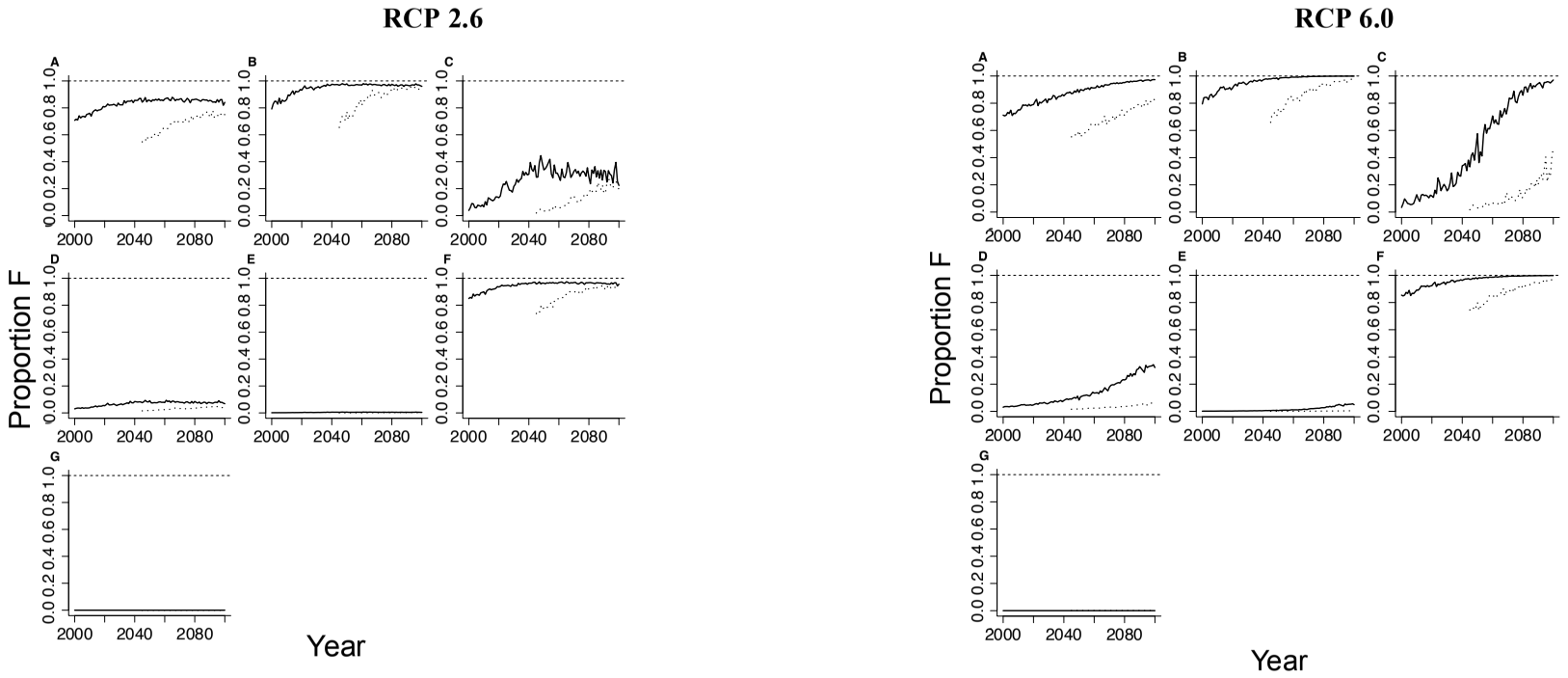

Figure A1.3(ii) Time series showing proportion of female hatchlings produced in Central America under RCP 2.6 and 6.0 using original reported Tpiv for sea turtles in A) North Mexico (Sandoval Espinoza 2012) B) Mexico (Batiz 1986), C, D & E) Costa Rica (Pacific) (Wibbels et al. 1998; McCoy et al. 1983; Brenes Arias et al. 2009) F) Costa Rica (Atlantic) (Spotila et al. 1987; Binckley et al. 1998). Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity

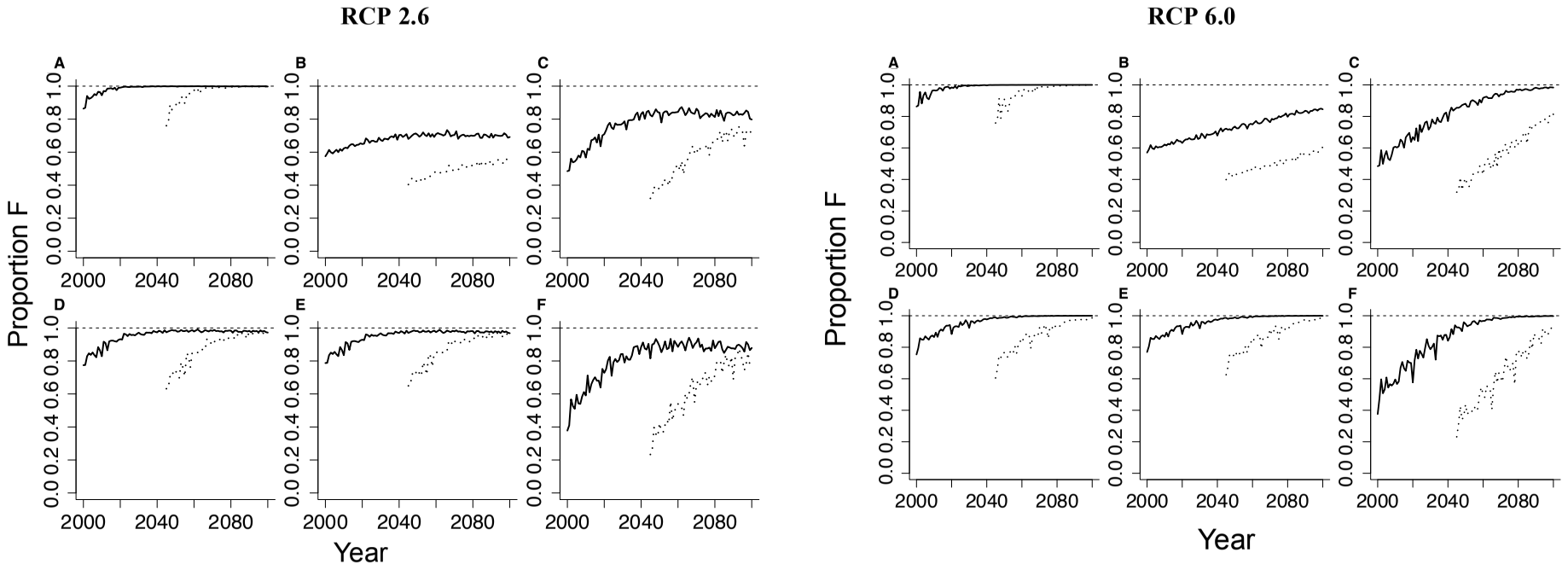

Figure A1.3(iii) Time series showing proportion of female hatchlings produced in South America under RCP 2.6 and 6.0 using the original reported T_{piv} for sea turtles in A) French Guiana (Rimblot et al. 1985) (R2) B & C) Suriname (Mrosovsky et al. 1984; Godfrey & Mrosovsky 2006), D, E & F) Brazil (Godfrey et al. 1999; Marcovaldi et al. 1997; Castheloge et al. 2018). Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity

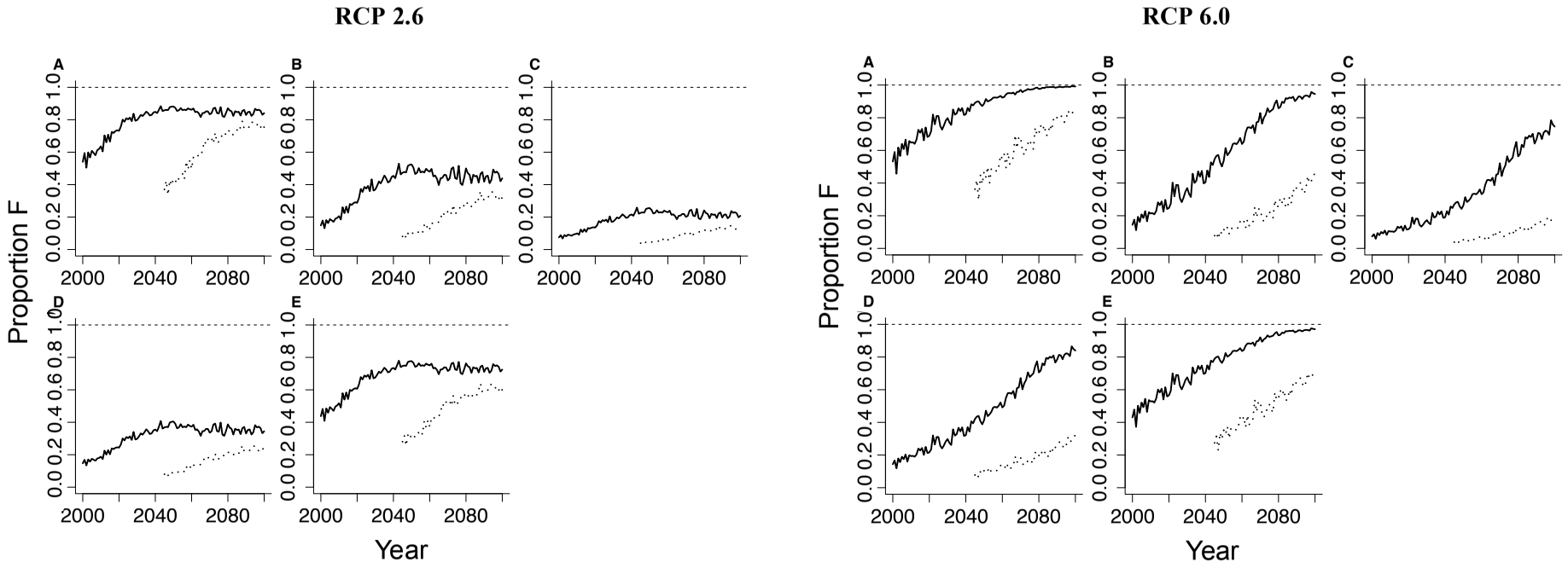

Figure A1.3(iv) Time series showing proportion of female hatchlings produced in the Mediterranean under RCP 2.6 and 6.0 using original reported T_{piv} for sea turtles in A) Spain(Chillon Segurado 2016) B) Greece (Mrosovsky et al. 2002) C & D) Turkey (Kaska et al. 1998) and E) Cyprus (Broderick et al. 2000). Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity.

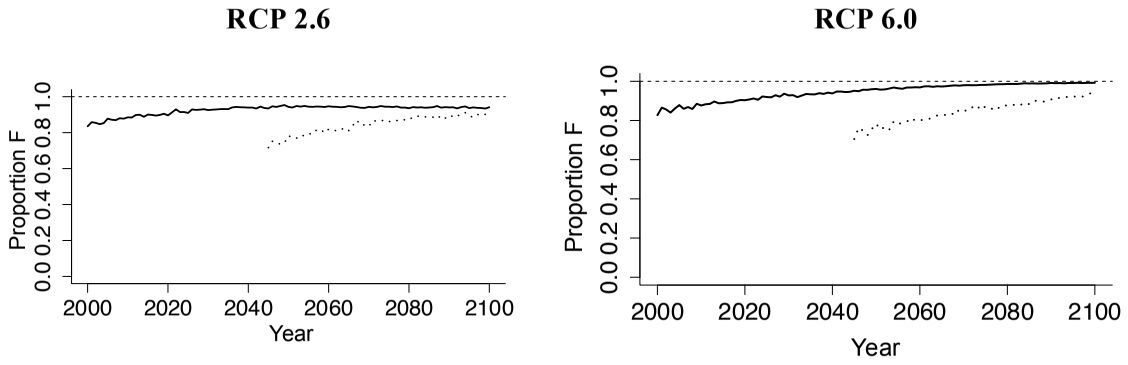

Figure A1.3(v) Time series showing proportion of female turtle hatchlings produced in Guinea Bisseau (Patrício et al. 2017) under RCP 2.6 and 6.0, using original reported T_{piv}. Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity.

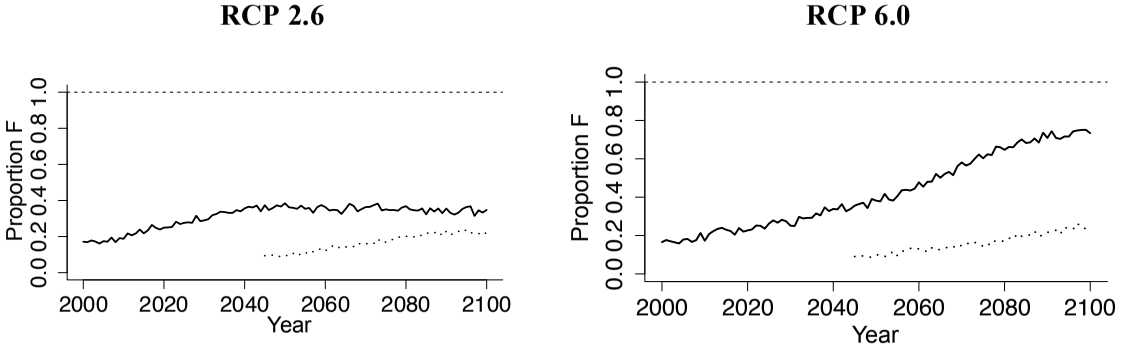

Figure A1.3(vi) Time series showing proportion of female turtle hatchlings produced in Taiwan (King et al. 2013) under RCP 2.6 and 6.0 using original reported T_{piv}. Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity.

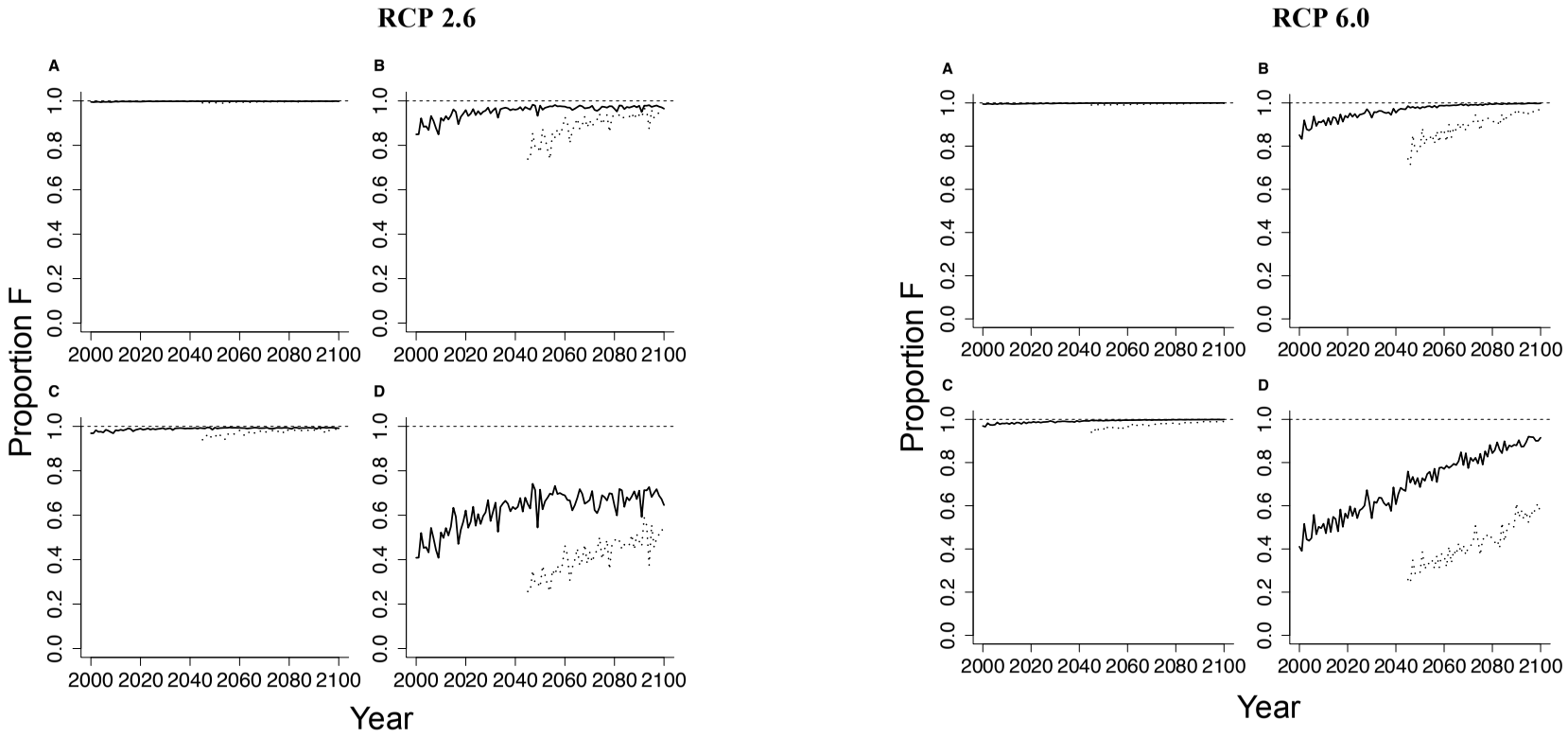

Figure A1.3vii) Time series showing proportion of female hatchlings produced in Australia under RCP 2.6 and 6.0 using original reported T_{piv}s for sea turtles in A) north Western Australia (Stubbs et al. 2014), B) northern Queensland (Dobbs et al. 2010) C) Western Australia (Woolgar et al. 2013) and D) Queensland (Georges et al. 1994). Solid line refers to offspring sex ratio, while dashed line represents OSR upon maturity.

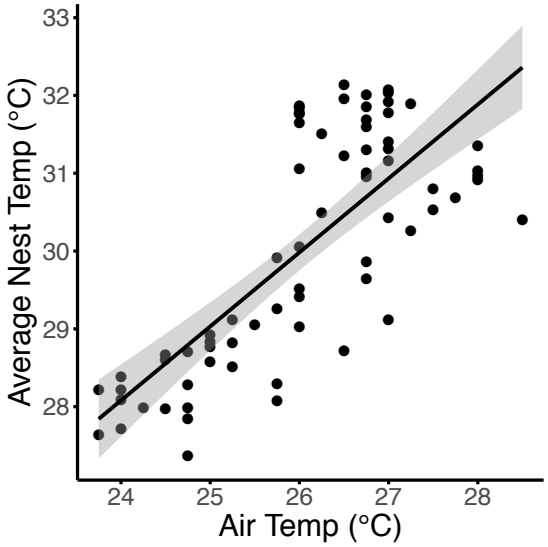

Figure A1.4: Scatterplot showing a significant relationship between the daily average air temperature and nest temperatures of 28 nests recorded in Boavista, Cape Verde, in 2017 (**$F_{1,69} = 92.833$, $p < 0.001$**).

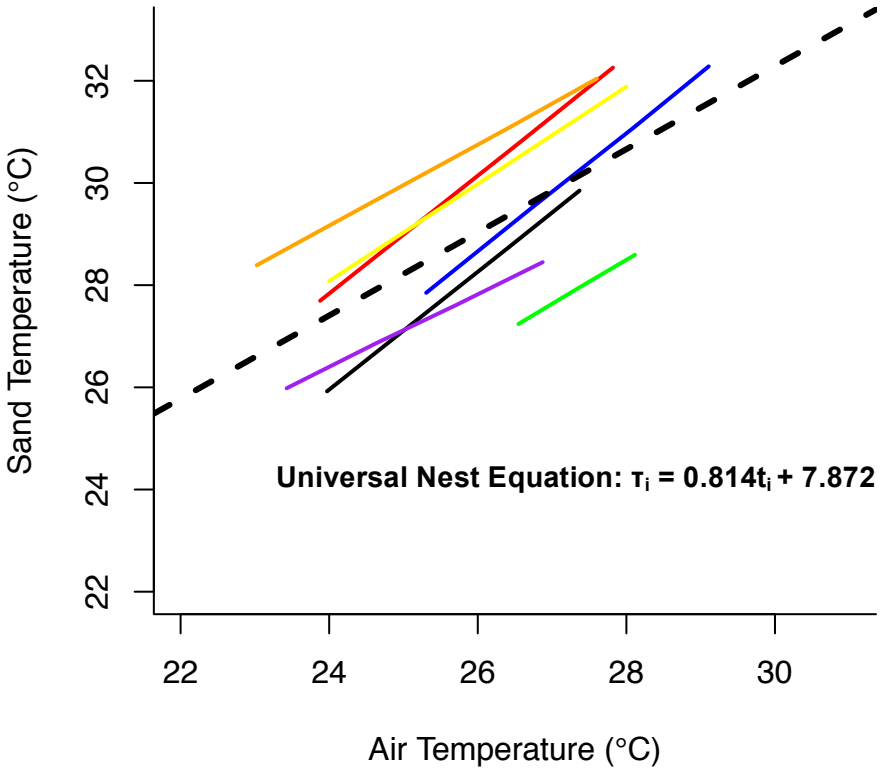

Figure A1.5: Figure adapted from (Esteban et al. 2016). To calculate a universal equation to predict nest temperatures, we took published data of the relationship between sand and air temperatures from other rookeries: Dark (orange) and light (purple) sand beaches in Ascencion Island, Diego Garcia (green), St Eustatius (blue), dark (red) and light (black) sand beaches in Cape Verde. We added information on the specific nest temperatures in relation to air temperature from our research in Cape Verde (yellow). Regressing all of this data gave us a universal equation for calculating nest temperature (black dashed line).

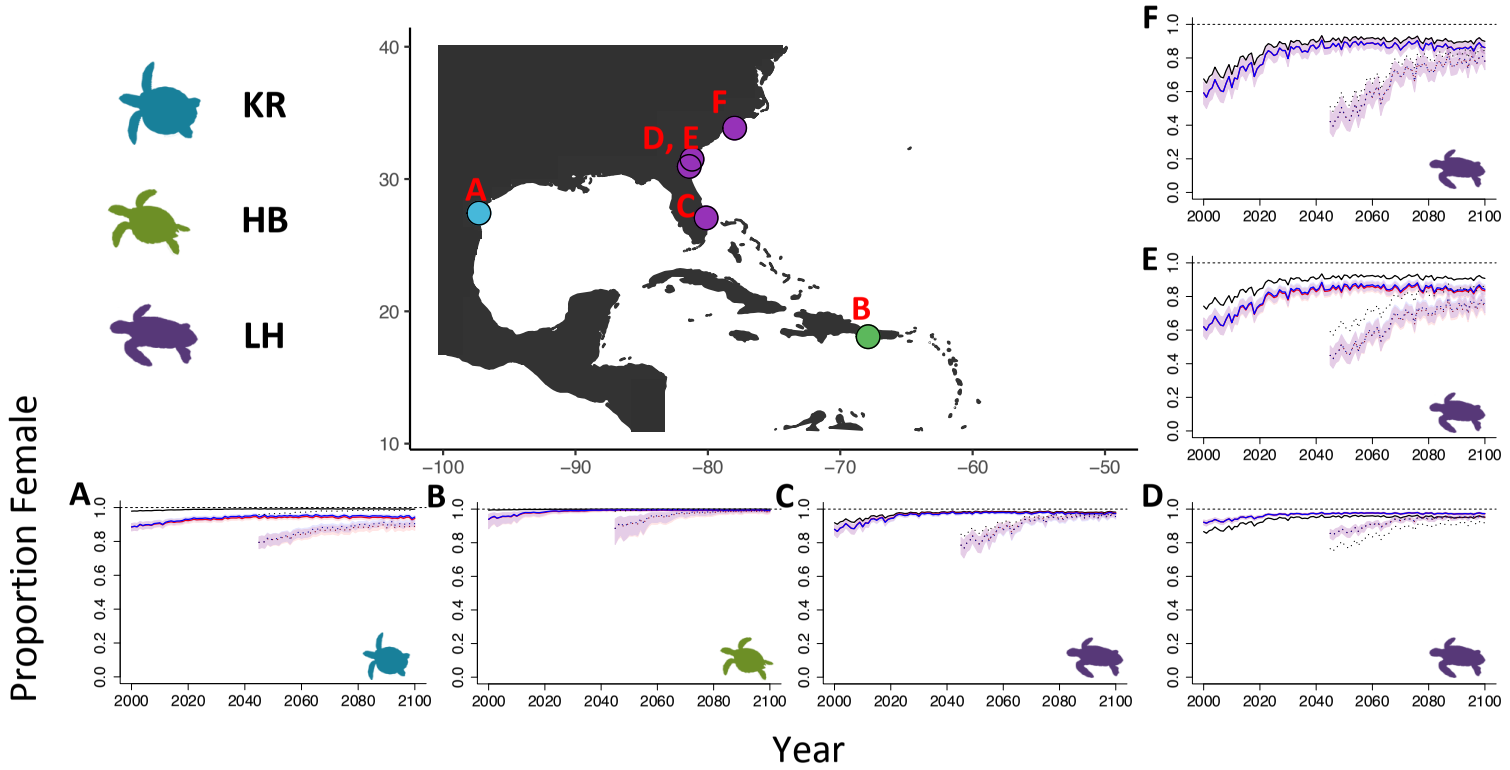

Figure A1.6(i) Time series showing proportion of female hatchlings produced at rookeries in North America and the Caribbean under RCP 2.6, for Kemp's ridley (KR), hawksbill (HB) and loggerhead (LH) sea turtles in A) Texas (LeBlanc et al. 2012) B) Puerto Rico (Mrosovsky et al. 2009), C) Florida (Mrosovsky 1988), D & E) Georgia (LeBlanc et al. 2012; Mrosovsky 1988) and F) North Carolina (Mrosovsky 1988). Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios.

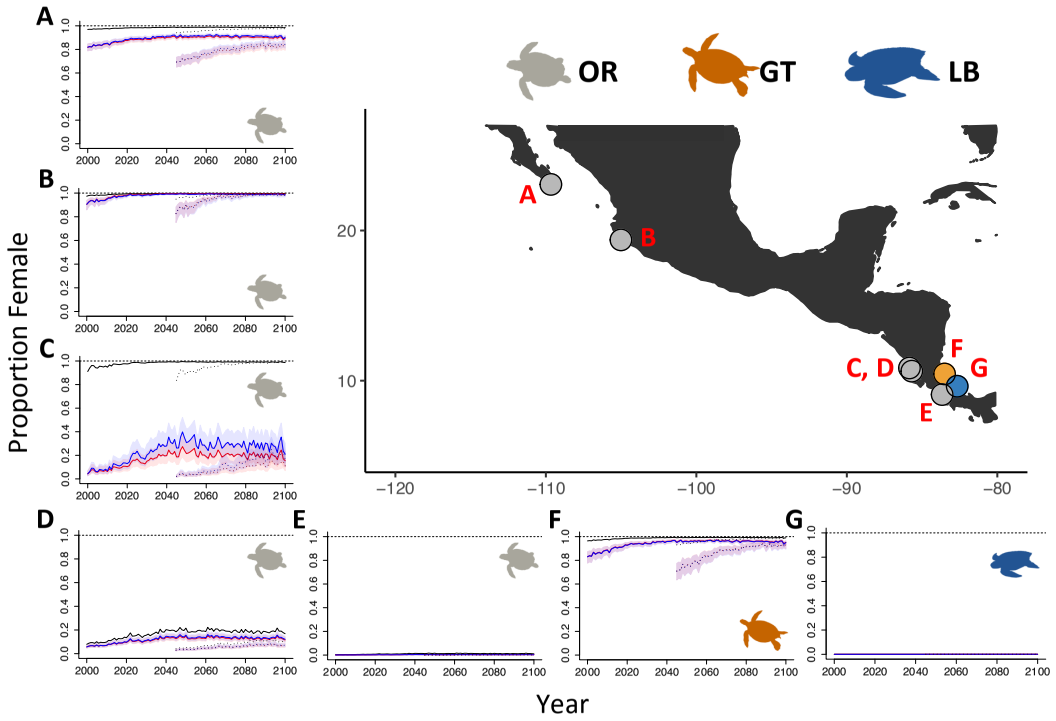

Figure A1.6(ii) Time series showing proportion of female hatchlings produced in Central America under RCP 2.6 for olive ridley (OR), green (GR) and leatherback (LB) in A) North Mexico (Sandoval Espinoza 2012) B) Mexico (Batiz 1986), C, D & E) Costa Rica (Pacific) (Wibbels, Rostal, et al. 1998; McCoy et al. 1983; Brenes Arias et al. 2009) F) Costa Rica (Atlantic) (Spotila et al. 1987; Binckley et al. 1998). Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios

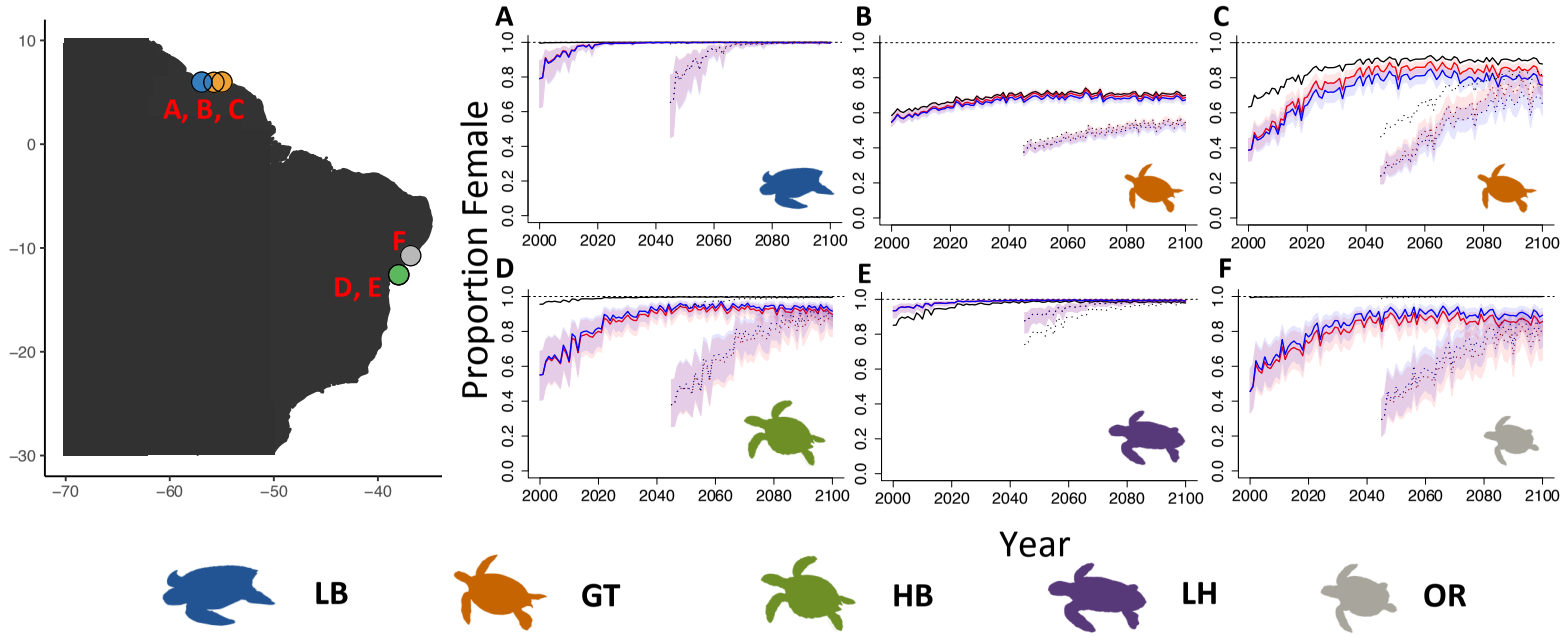

Figure A1.6(iii) Time series showing proportion of female hatchlings produced in South America under RCP 2.6 for leatherback (LB), green (GR), hawksbill (HB), loggerhead (LH) and olive ridley (OR) sea turtles in A) French Guiana (Rimblot et al. 1985) (R2) B & C) Suriname (Mrosovsky et al. 1984; Godfrey & Mrosovsky 2006), D, E & F) Brazil (Godfrey et al. 1999; Marcovaldi et al. 1997; Castheloge et al. 2018). Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios

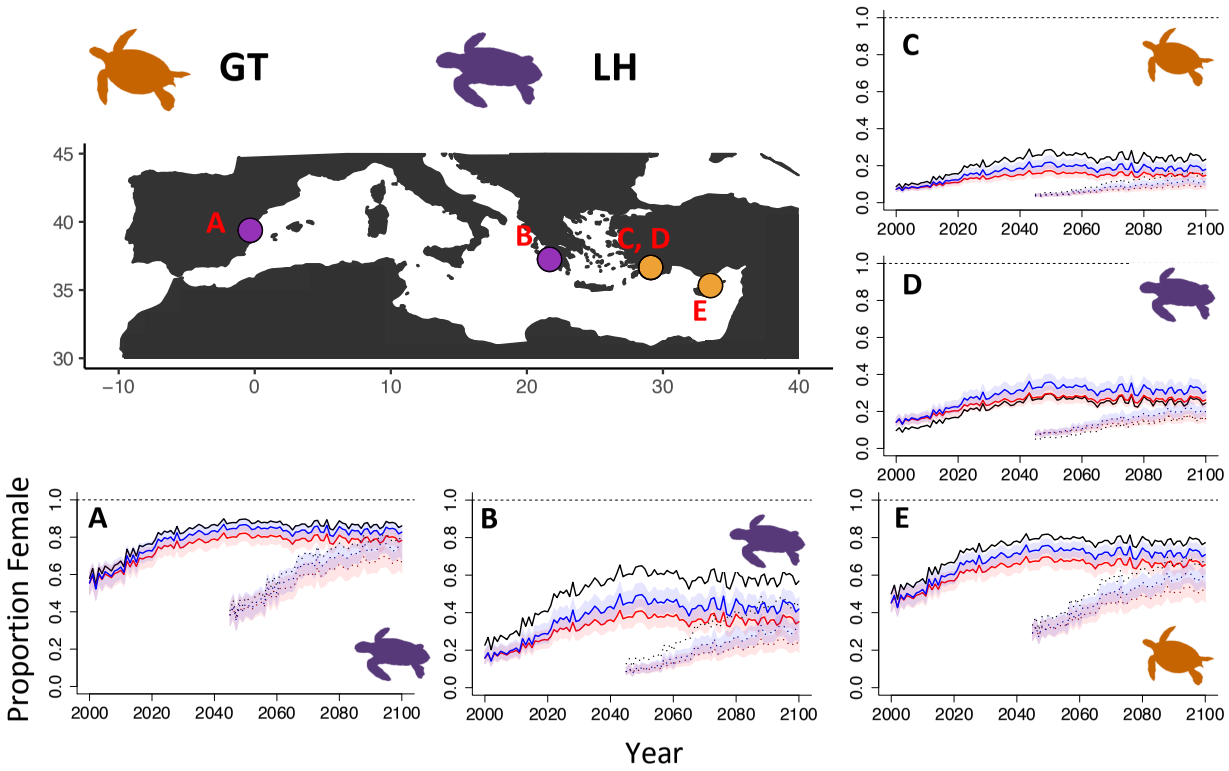

Figure A1.6(iv) Time series showing proportion of female hatchlings produced in the Mediterranean under RCP 2.6 for green (GR) and loggerhead (LH) in A) Spain (Chillon Segurado 2016) B) Greece (Mrosovsky et al. 2002) C & D) Turkey (Kaska et al. 1998) and E) Cyprus (Broderick et al. 2000). Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios

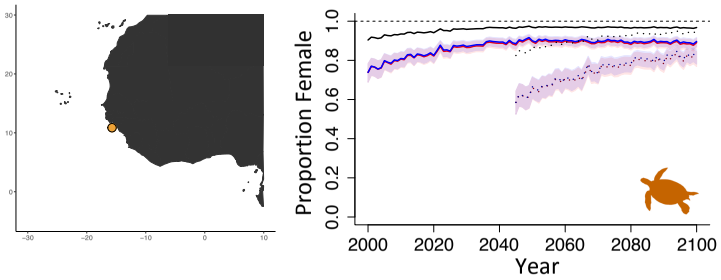

Figure A1.6(v) Time series showing proportion of female green turtle hatchlings produced in Guinea Bisseau (Patrício et al. 2017) under RCP 2.6. Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios

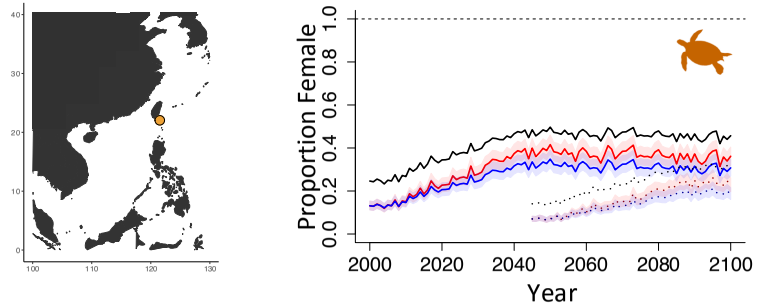

Figure A1.6(vi) Time series showing proportion of female green turtle hatchlings produced in Taiwan (King et al. 2013) under RCP 2.6. Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios.

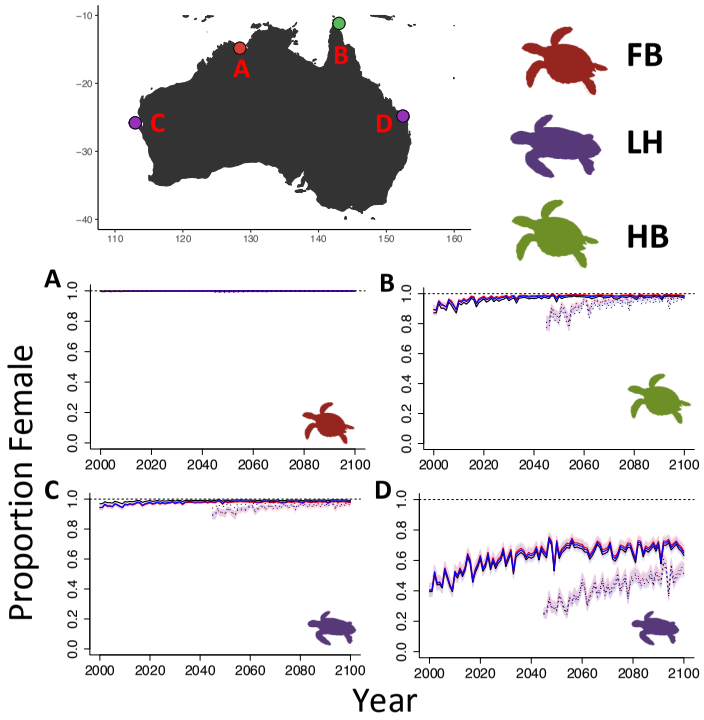

Figure A1.6vii) Time series showing proportion of female hatchlings produced in Australia under RCP 2.6 for flatback (FB), loggerhead (LH) and hawksbill (HB) sea turtles in A) north Western Australia (Stubbs et al. 2014), B) northern Queensland (Dobbs et al. 2010) C) Western Australia (Woolgar et al. 2013) and D) Queensland (Georges et al. 1994). Black lines project proportion female offspring under T_{piv} of 29 °C, red lines are projections under a plastic scenario (with 95% CI) and blue lines projections under a heritable scenario (with 95% CI). Dashed lines correspond to OSRs for the three scenarios.

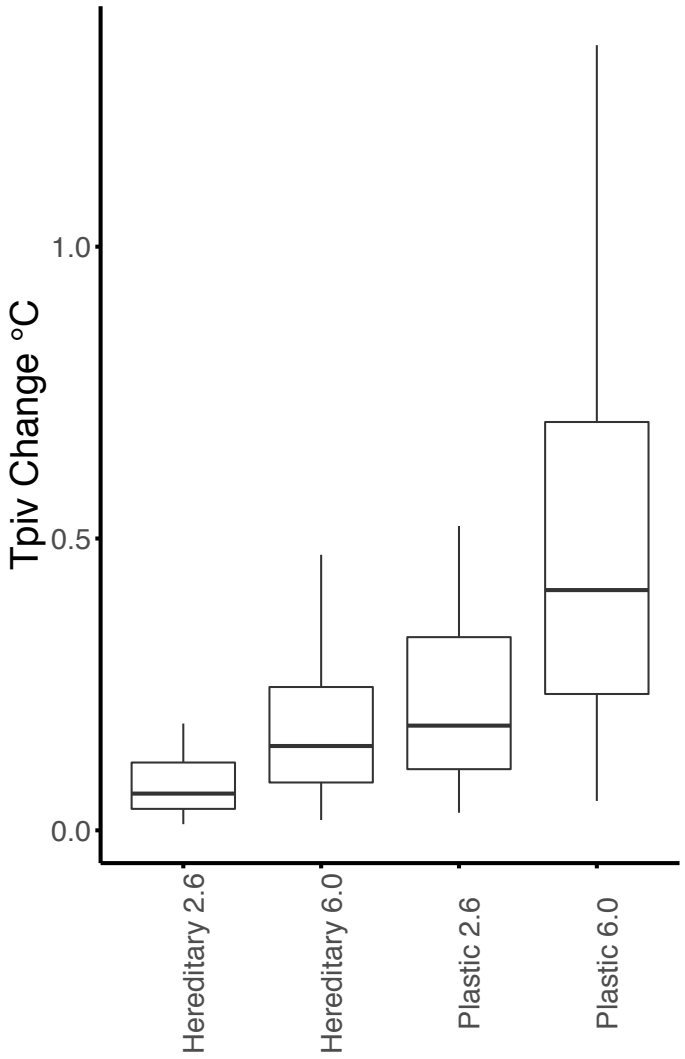

Figure A1.7: Boxplots showing the change in pivotal temperature between our two adaptive scenarios. There was a greater shift in T_{piv} under plastic conditions for both RCP 2.6 ($df = 33.553$, $t = -5.064$, $p < 0.001$) and RCP 6.0 ($df = 33.553$, $t = -4.558$, $p < 0.001$).

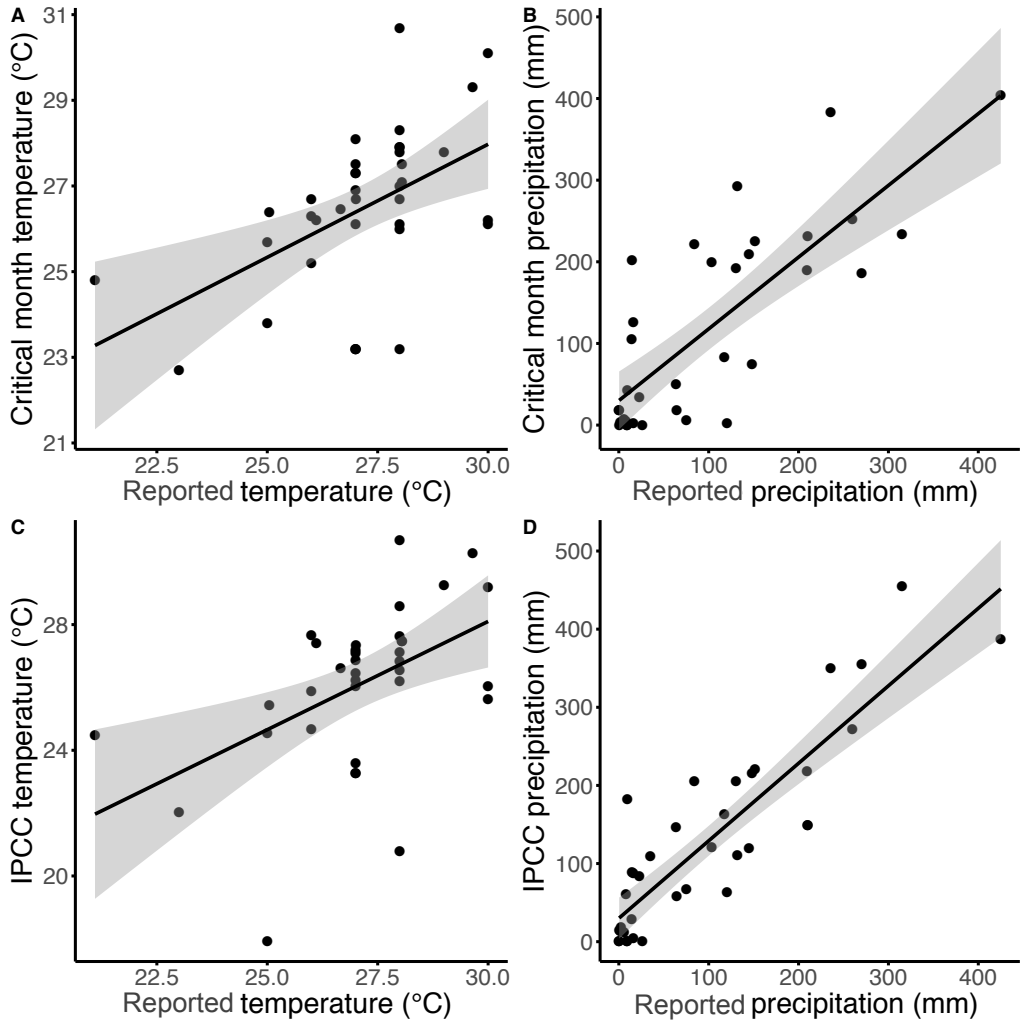

Figure A1.8: Scatterplots showing a strong correlation between monthly average air temperatures and total precipitation reported online, and that downloaded from NOAA for both A) Average temperature during the critical month ($F_{1,35}$ = 11.923, $p < 0.001$) B) Total rainfall during the critical month ($F_{1,32}$ = 54.300, $p < 0.001$), C) IPCC baseline temperatures ($F_{1,35}$ = 10.645, $p < 0.001$) D) IPCC total precipitation baseline ($F_{1,35}$ = 122.13, $p<0.001$)

Table A1.1: Examples of studies that use thermal proxies to indirectly estimate offspring sex ratios, and the origin of the T_{piv} used to estimate this.

Species	**Location**	**T_{piv}**	**Source of T_{piv}**
Green Turtle (Esteban et al. 2016)	Chagos Archipelago	29	Review(Ackerman 1997)
Green Turtle (Broderick et al. 2001)	Ascension Island	29	Review(Ackerman 1997)
Green Turtle (Booth & Freeman 2006)	Heron Island, Australia	27.5	T_{piv} previously calculated for population(Booth & Astill 2001)
Hawksbill (Esteban et al. 2016)	Chagos Archipelago	29	Review(Ackerman 1997)
Hawksbill (Glen & Mrosovsky 2004)	Antigua	29.2	Review(Mrosovsky & Pieau 1991)
Loggerhead (Laloë et al. 2014)	Cape Verde	29, 28.8 and 29.2	Mathematical modelling - Fit three different T_{piv} and kept 29 °C
Loggerhead (Zbinden et al. 2007)	Zakynthos	29.3	T_{piv} previously calculated for population(Mrosovsky et al. 2002)
Loggerhead (Öz et al. 2004)	Turkey	29	T_{piv} previously calculated for population(Kaska et al. 1998)
Loggerhead (Hanson et al. 1998)	Florida	29	T_{piv} previously calculated for population(Mrosovsky 1988)

Table A1.2: Data included within latitudinal and environmental models, including the originally reported and re-calculated T_{piv} and TRT. Merged column describes the data used within analysis – recalculated values were used where possible. Projected represents whether these rookeries were included within IPCC projection scenarios.

			Original Reported		Re-calculated		Merged		
Country	**Latitude**	**Longitude**	**T_{piv}**	**TRT**	**T_{piv}**	**TRT**	**T_{Piv}**	**TRT**	**Projected**
Flatback									
Australia (Stubbs et al. 2014)	-14.822	128.394	29.4	NA	29.85	3.22	29.85	3.22	Yes
Green									
Suriname (Mrosovsky et al. 1984)	5.961	-55.761	28.75	NA	29.06	9.2	29.06	9.2*	Yes
Turkey (Kaska et al. 1998)	36.664	29.107	28.9	NA	29.13	4.79	29.13	4.79	Yes
Cyprus (Broderick et al. 2000)	35.334	33.491	29.2	NA	NA	NA	29.2	NA	Yes
Ascension Island (Godley et al. 2002)	-7.946	-14.355	28.8	NA	28.81	3.49	28.81	3.49	No
Suriname (Godfrey & Mrosovsky 2006)	5.994	-54.986	29.4	2.1	29.35	3.39	29.35	3.39	Yes
Costa Rica (Spotila et al. 1987)	10.448	-83.506	28.5-30	NA	29.73	2.76	29.73	2.76	Yes
Guinea-Bissau (Patrício et al. 2017)	10.867	-15.716	29.4	3.8	29.4	3.8	29.4	3.8	Yes
Taiwan (King et al. 2013)	22.039	121.521	29	NA	29.4	5.16	29.4	5.16	Yes
Australia (Stubbs & Mitchell 2018)	-21.812	114.088	29.2	2.5	29.2	2.71	29.2	2.71	No
Hawksbill									
Puerto Rico (Mrosovsky et al. 2009)	18.082	-67.892	29.6	1.3	29.6	1.3	29.6	1.3	Yes
Antigua (Mrosovsky et al. 1992)	16.999	-61.797	29.2	NA	29.28	1.04	29.28	1.04	No
Brazil (Godfrey et al. 1999)	-12.574	-38.004	29.65	NA	29.5	1.599	29.5	1.599	Yes
Australia (Dobbs et al. 2010)	-11.169	143.015	29.2	NA	29.1	3.09	29.1	3.09	Yes

Kemp's Ridley									
USA(LeBlanc et al. 2012)	27.425	-97.296	30	> 2.4	29.68	4.35	29.68	4.35	Yes
Mexico (Shaver et al. 1988)	23.394	-106.523	30.2	NA	NA	NA	30.2	NA	No
Leatherback									
French Guiana (Rimblot et al. 1985)	5.981	-56.932	28.75 - 29.75	NA	29.68	0.13	29.68	0.13	Yes
Costa Rica (Binckley et al. 1998)	9.631	-82.666	29.4	1	NA	NA	29.4	NA	Yes
Loggerhead									
USA (LeBlanc et al. 2012)	31.5	-81.2	28.9	4	29.14	4.09	29.14	4.09	Yes
Greece (Mrosovsky et al. 2002)	37.251	21.669	29.33	1.5	NA	NA	29.33	1.5	Yes
USA (Yntema & Mrosovsky 1982)	30.951	-81.413	30	NA	29.87	3.27	29.87	3.27	Yes
USA (Mrosovsky 1988)	33.857	-77.982	29.1	NA	29.17	3.44	29.17	3.44	Yes
USA (Mrosovsky 1988)	30.951	-81.413	28.5	NA	28.2	4.5	28.2	4.5	Yes
USA (Mrosovsky 1988)	27.059	-80.136	29.2	NA	29.1	3.22	29.1	3.22	Yes
South Africa (Maxwell et al. 1988)	-26.987	32.867	29.7	NA	29.05	0.964	29.05	0.964	Yes
Brazil (Marcovaldi et al. 1997)	-12.574	-38.004	29.2	NA	29.13	1.76	29.13	1.76	Yes
Turkey (Kaska et al. 1998)	36.663	29.107	28.9	NA	28.59	5.03	28.59	5.03	Yes
Australia (Georges et al. 1994)	-24.805	152.441	29.2	2	28.95	5.06	28.95	5.06	Yes
Australia(Woolgar et al. 2013)	-25.798	112.979	29	0.67	NA	NA	29	0.67	Yes
Spain (Chillon Segurado 2016)	39.382	-0.332	29.1	NA	NA	NA	29.1	NA	No
Olive Ridley									
Costa Rica (McCoy et al. 1983)	10.633	-85.683	30	NA	29.76	4.41	29.76	4.41	Yes
Costa Rica (Wibbels et al. 1998)	10.846	-85.840	30.8	NA	30.79	1.9	30.79	1.9	Yes

Colombia (Martinez & Paez 2000)	5.4452	-77.4201	30.5	2.9	31.5	4.54	31.5	4.54	No
Mexico (Batiz 1986)	19.375	-105.011	29.9	NA	29.8	2.07	29.8	2.07	Yes
Costa Rica (Brenes Arias et al. 2009)	9.075	-83.667	29.54	NA	NA	NA	29.54	NA	No
Mexico (Sandoval Espinoza 2012)	23.071	-109.666	29.95	5.8	31.08	4.75	31.08	4.75	Yes
Brazil (Castheloge et al. 2018)	-10.740	-36.850	30.7	1.8	30.7	1.8	30.7	1.8	Yes

Table A1.3: T_{piv} change in response to climate change under the two adaptive scenarios.

		Min	Max	Mean
RCP 2.6	**S2 (Plastic)**	0.030	0.521	0.214 ± 0.137
	S2 (Hereditary)	0.011	0.183	0.075 ± 0.048
RCP 6.0	**S3 (Plastic)**	0.050	1.345	0.489 ± 0.348
	S3 (Hereditary)	0.017	0.472	0.172 ± 0.122

Appendix 2: Supplementary Material for Chapter 2

1. Hormone Extraction Protocol

Both testosterone (T) and oestradiol (E_2) were extracted from adult and hatchling plasma samples following commercial ELISA kit protocols (E_2: Catalogue # ADI-900-174, T: Catalogue # ADI-900-065, ENZO Life Sciences). Anhydrous diethyl ether was added at a ratio of 5:1 to a 40-200 µl plasma sample. After homogenising and snap freezing, the diethyl ether fraction was decanted into a fresh test tube containing either 0.5 or 1 ml of distilled water and homogenised once more. The resulting organic phase was removed and evaporated for two hours using a speed vacuum. Samples were then rehydrated using 250 µl of appropriate assay buffer from the ELISA kits, and frozen until assayed. Yolk hormones were extracted following the protocol from Schwabl (1993) with the exception of the final hexane phase, which was not conducted. After extraction, samples were reconstituted in 250 µl of appropriate assay buffer.

Extraction efficiencies were determined for both hormones by dividing either adult plasma samples (E_2: n = 6, T: n = 5) or yolk samples (E_2: n = 6, T: n = 5) into two aliquots. One of these was spiked with a known concentration of hormone (E_2: 272 pg/ml, T: 400 pg/ml,) prior to extraction. Efficiency was determined by calculating the difference between the spiked and non-spiked samples compared to the known spike quantity (Plasma: E_2: 54.5 ± 10.5 (SE) %, T: 43.9 ± 2.8 (SE) %, Yolk: E_2: 77.9 ± 6 (SE)%, T: 120.2 ± 9.8 (SE) %).

Serially diluted standards of known hormone concentration were prepared (E_2 = 7, T = 5) according to the kit's protocol, producing standard curves ranging from 1000 – 15.6 pg/ml (E_2) and 2000 – 7.81 pg/ml (T). Samples were run in duplicate, and hormone concentrations were calculated using a curve-fitting program (MARS). All yolk samples were run on a single plate, with average intra-assay coefficients of variation (CV) being 10.63 ± 1.24 (SE) % for E_2 and 9.89 ± 1.32 (SE) % for T. Hatchling plasma samples were run across six plates for each hormone. Intra-assay CVs were 13.5 ± 1.3 (SE) % for E_2 and 17.05 ± 2.9 (SE)% for T.

Supplementary Analysis:

1. Circulating E_2 and T within adult plasma

To support our observation that the levels of circulating E_2 correlate with T within adult plasma, we augment our dataset by also including samples collected from nesting turtles in the same location in 2016 (2016: n = 75, total including this dataset: n = 92). With this larger sample size we strengthen the relationship observed within this study, showing a strong positive correlation between the two hormones ($F_{1,91}$ = 93.527, $p < 0.001$).

2. Relationship between metabolic heat and incubation duration

There is a well-known link between heat and developmental rates (Monsinjon et al. 2017). Thus we quantified how metabolic heat influenced incubation duration within this study. Clutch size positively correlated with temperature, likely as a result of metabolic heat production (Fig. A2.6A, $F_{1,26}$ = 4.42, p = 0.05). Elevated metabolic heat positively correlated with embryonic development rates as expected, and resulted in shorter incubation durations (Fig. A2.6B, $F_{1,26}$ = 4.31, p = 0.05). Based on the slope estimate of this model, if metabolic heat was the sole determinant of variation in incubation duration within our study clutches, the range of temperatures recorded should cause the incubation duration to vary by a maximum of three days (95% CI = 2.4, mean: 56.63 ± 0.14 (SE), min = 55.34; max = 58.44 days). Yet, observed incubation durations ranged across seven days, from 54 to 61 days (mean: 56.57 ± 0.37 (SE)), implying that factors other than metabolic heat production associated with clutch size contributed to variation in rates of embryonic development – in this case, the most likely explanation is intrinsic egg characteristics.

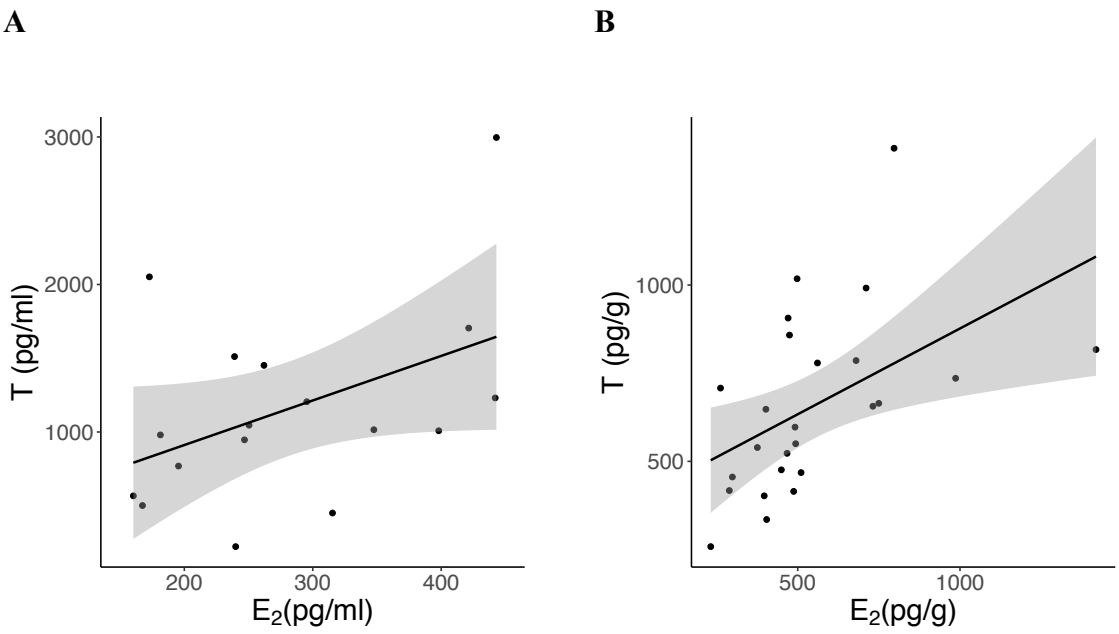

Figure A2.1: Scatterplots showing a significant, positive correlation exists between E_2 and T in both A) adult plasma ($F_{1,16} = 4.608$, $p = 0.048$) and B) egg yolks ($F_{1,23} = 7.338$, $p = 0.013$)

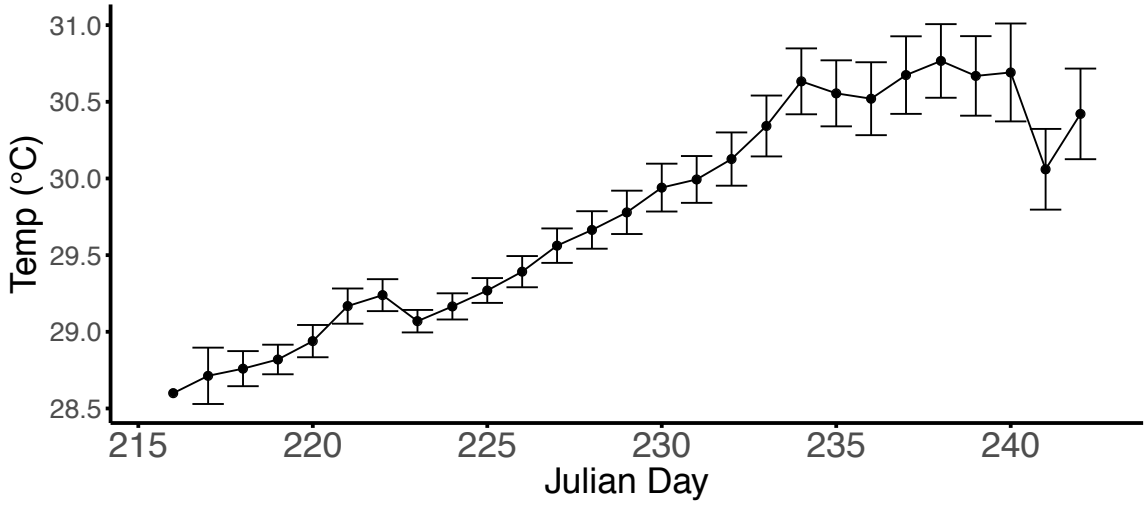

Figure A2.2: Time series showing mean temperature ± 95% CI of 28 clutches of eggs through the thermosensitive period of incubation in 2017

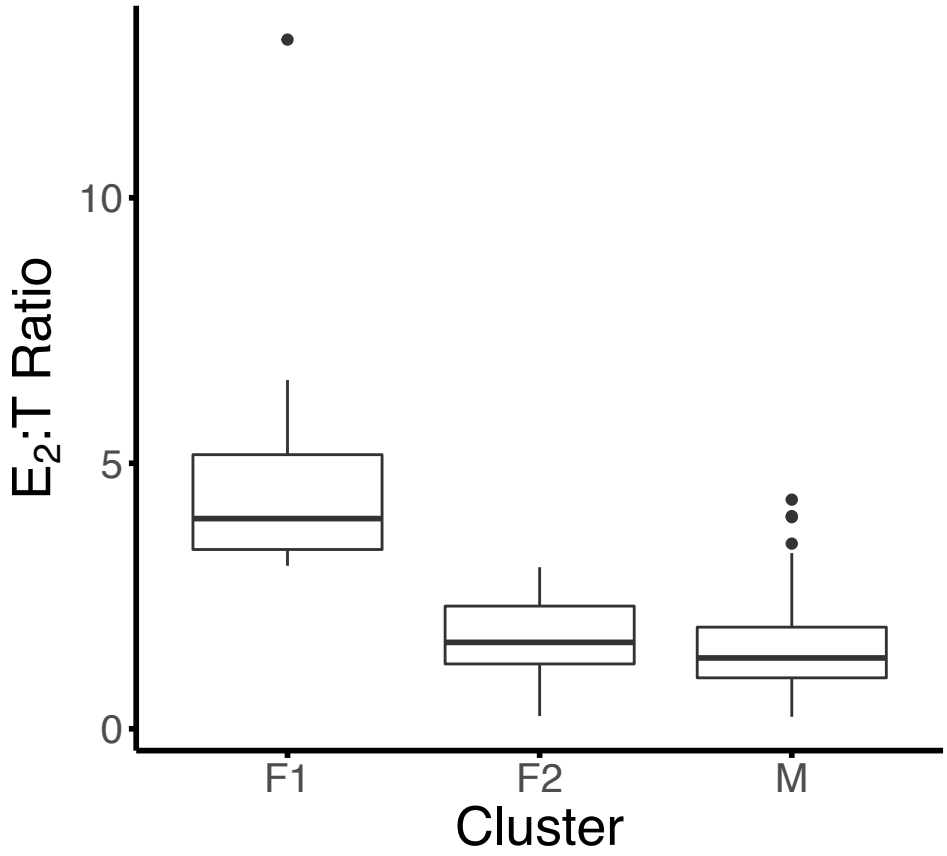

Figure A2.3: Differences in mean (with 95% CI) E_2:T ratio between the three different clusters identified by APC clustering. Clusters F1 and F2 are assumed to be female from their short incubation duration, and differ in their E_2:T ratios. Cluster M is produced under the long incubation durations characteristic of male offspring, and have low E_2:T ratios.

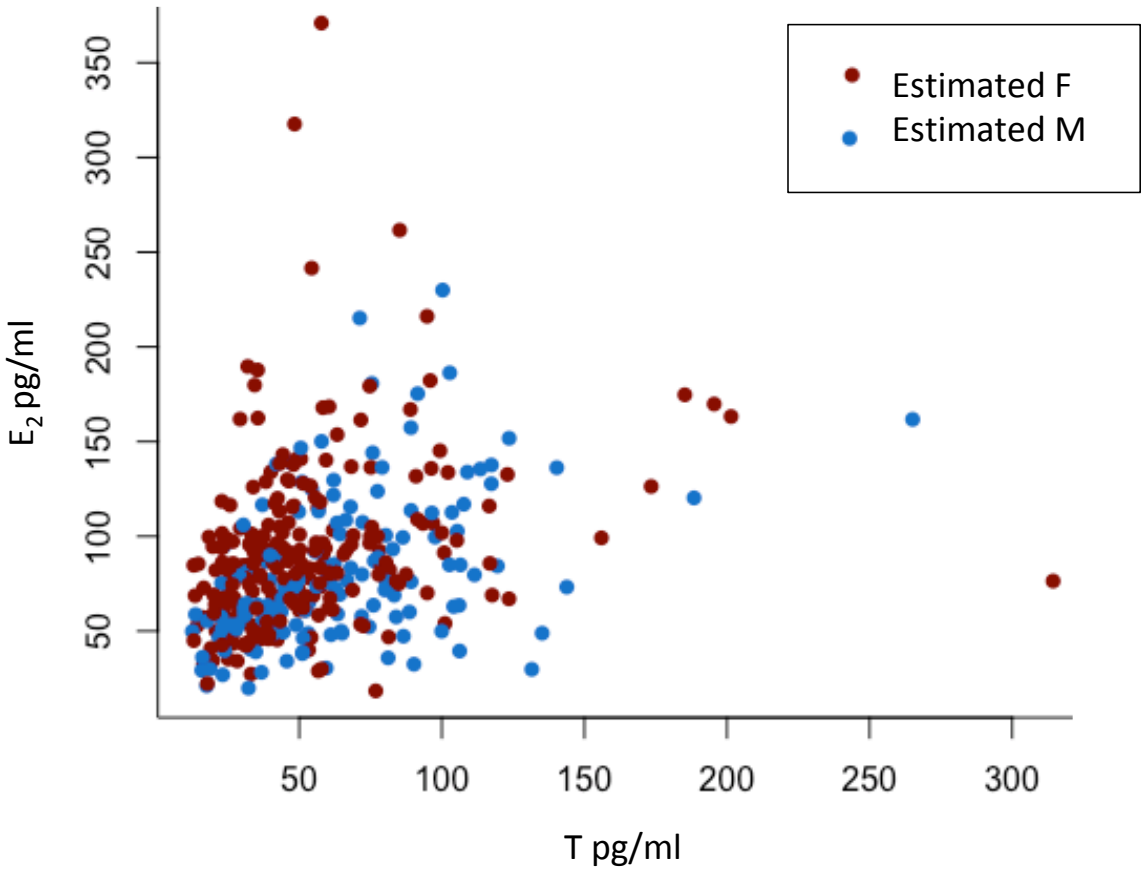

Figure A2.4: Scatterplot showing raw E_2 and T concentrations of hatchlings estimated as male and female by the APC clustering method.

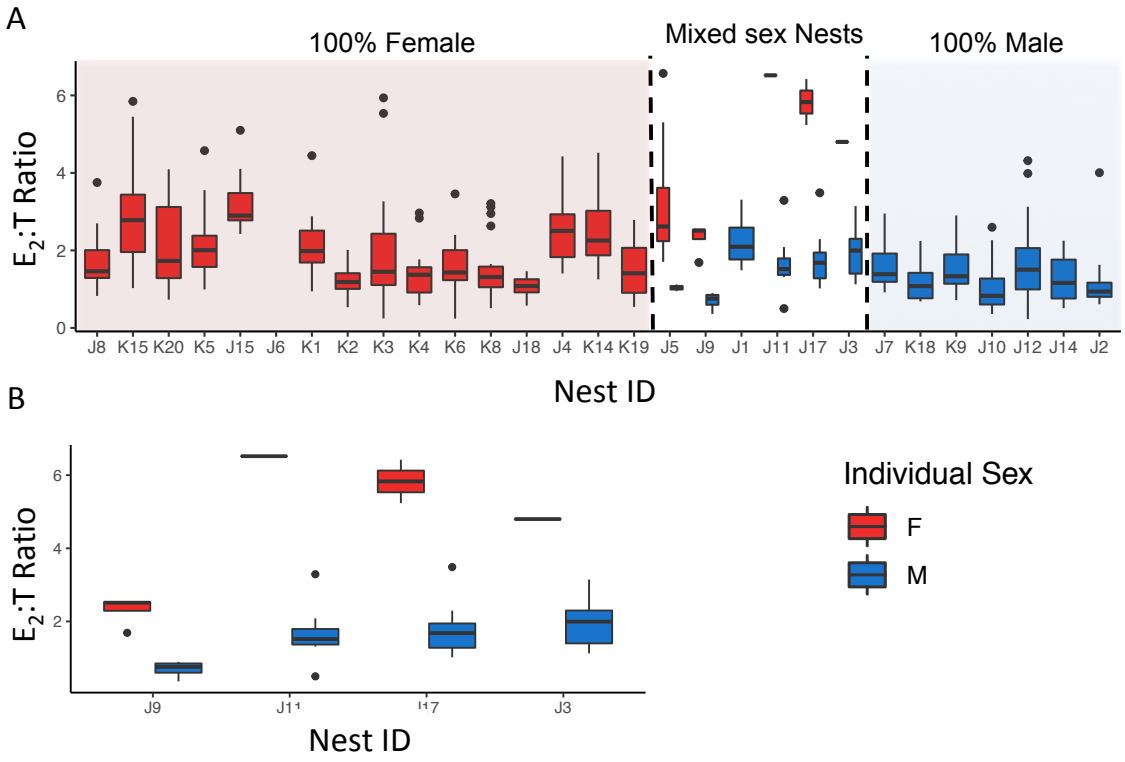

Fig A2.5: A) Boxplots summarising the E_2:T ratios of hatchlings by nest (ordered by incubation duration, shortest to longest) and by assumed sex, as estimated by APC clustering. B) Focusing on mix-sex nests, there is a clear cleavage between the E_2:T ratios of estimated male and estimated female hatchlings.

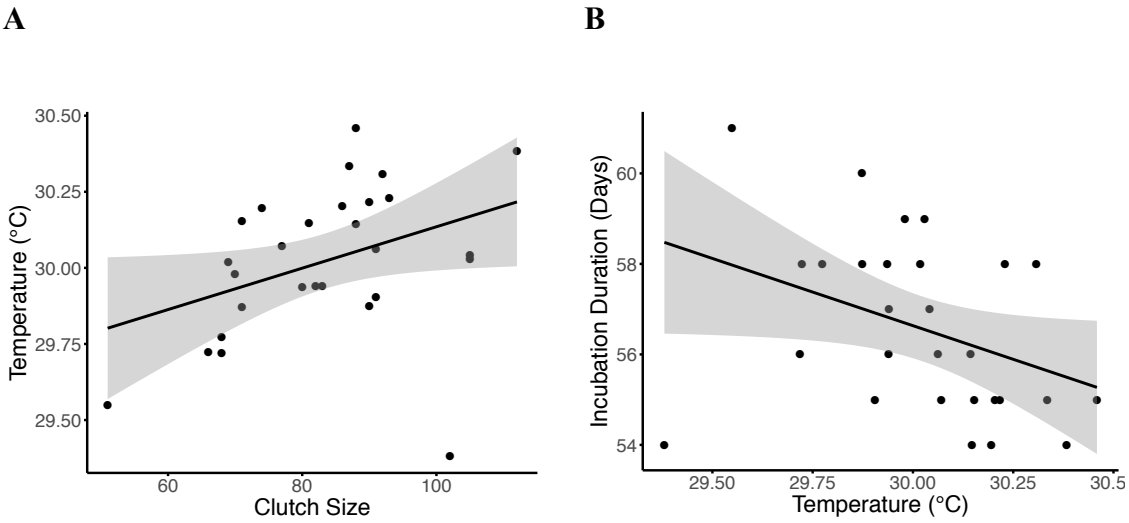

Figure A2.6: Scatterplots showing characteristic relationships between clutch size, temperature and rate of development were observed in study clutches. A) There was a significant positive relationship between clutch size and the temperature of the nest ($F_{1,26} = 4.42$, $p = 0.05$); B) Temperature had a negative influence on incubation duration ($F_{1,26} = 4.31$, $p = 0.05$)

Table A2.1: Adult and hatchling plasma E_2 and T concentrations and the E_2:T ratio recorded in populations of sea turtles globally. Values from this study in bold. *Measurements taken from beginning of nesting season only. **Measurements taken from free-swimming, reproductively active female turtles at varying points within the nesting season.

Location	Species	n	Category	Sex	E_2 pg/ml	T pg/ml	Ratio
Adults							
Oman (Al-Habsi et al. 2006)	Green	22	nesting	F	undetected	420 ± 40 (SE)	NA
Costa Rica (Rostal et al. 2001)	Leatherback	32	nesting*	F	190 ± 16.8 (SE)	10180 ± 77000 (SE)	NA
Costa Rica (Rostal et al. 1996)	Leatherback	13	nesting*	F	53.30 ± 6.54 (SE)	2224 ± 280	NA
Australia (Dobbs et al. 2007)	Hawksbill	95	nesting	F	0 - 119 (range)	0-7520 (range)	NA
Florida (Myre et al. 2016)	Loggerhead	38	free-swimming**	F	3.2 - 3723 (range)	50 - 12900 (range)	NA
Cape Verde	**Loggerhead**	**26**	**nesting**	**F**	**235.8 ± 22.7 (SE)**	**1148.5 ± 148.6 (SE)**	**0.32 ± 0.05 (SE)**
Hatchlings							
China (Xia et al. 2011)	Green	16	post-emergence	M	132 ± 37 (SD)	186 ± 58 (SD)	0.788 ± 0.338 (SD)
China (Xia et al. 2011)	Green	14	post-emergence	F	205 ± 50 (SD)	105 ± 30 (SD)	2 ± 0.438 (SD)
Japan (Kobayashi et al. 2017)	Loggerhead	90	post-emergence	NA	0 - 50.2 (range)	9.2 - 300.2 (range)	0.01 - 1.24 (range)
North Carolina/ Florida (Gross et al. 1995)	Loggerhead	17	post-emergence	M	106 ± 15 (SD)	215 ± 38 (SD)	0.6 ± 0.1 (SD)
North Carolina/ Florida (Gross et al. 1995)	Loggerhead	11	post-emergence	F	198 ± 44 (SD)	76 ± 13 (SD)	2.7 ± 0.4 (SD)
Cape Verde	**Loggerhead**	**151**	**post-emergence**	**M**	**81.66 ± 3.16 (SE)**	**63.63 ± 2.9 (SE)**	**1.52 ± 0.06 (SE)**
Cape Verde	**Loggerhead**	**253**	**post-emergence**	**F**	**92.93 ± 3.06 (SE)**	**52.53 ± 2.34 (SE)**	**2.22 ± 0.09 (SE)**

Appendix 3: Supplementary Material from Chapter 3

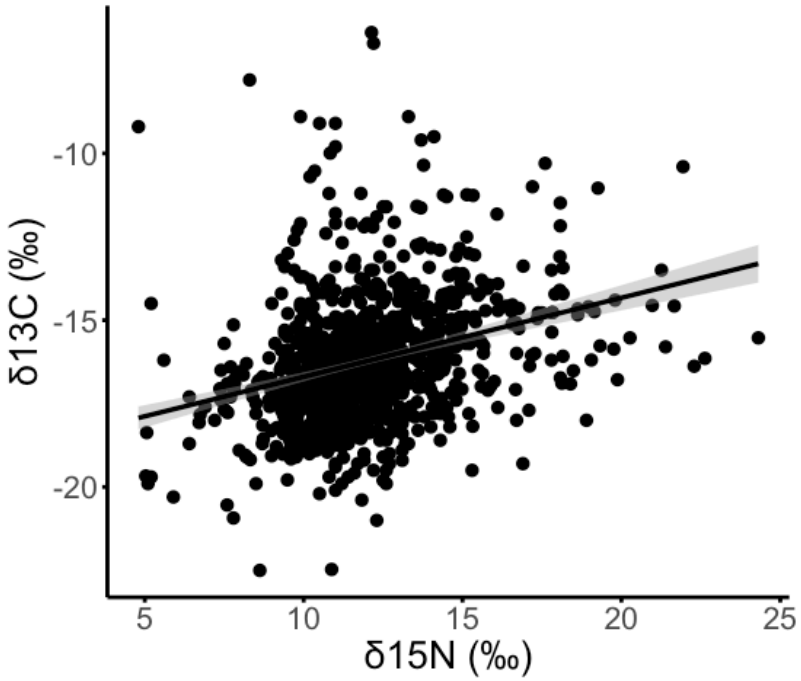

Figure A3.1: Scatterplot showing a significant correlation between $\delta^{15}N$ and $\delta^{13}C$ values of skin samples from adult nesting females. ($F_{1,924} = 84.603$, $p < 0.001$)

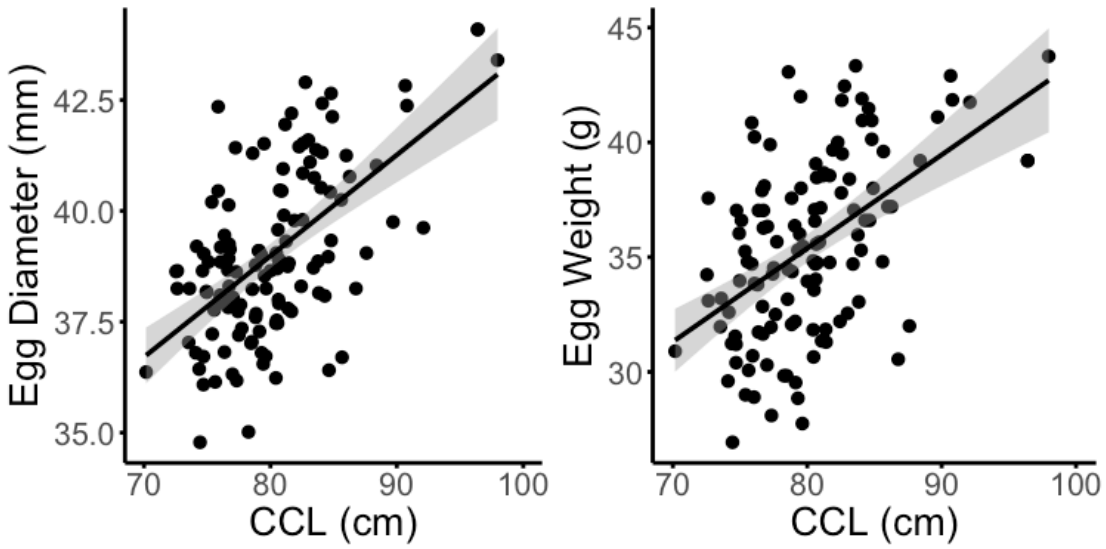

Figure A3.2: Scatterplots showing maternal size (CCL) significantly correlated with egg size ($F_{1,99} = 37.672$, $p < 0.001$) and weight ($F_{1,119} = 54.319$, $p < 0.001$).

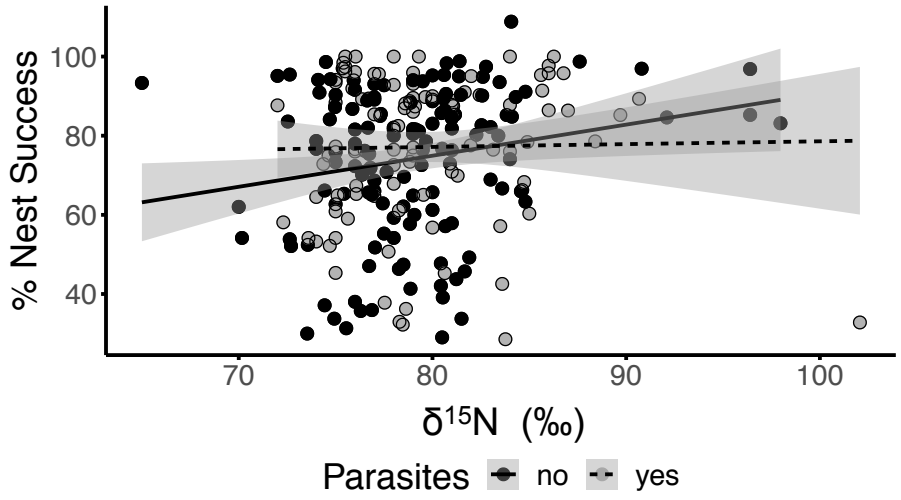

Figure A3.3: Scatterplot showing a significant interaction between the $\delta^{15}N$ value and infection status of a nesting female turtle, and the success rate of a nest ($F_{1,126} = 10.731$, $p = 0.001$). While plots is bivariate, statistics reported are from final reported multiple regression models.

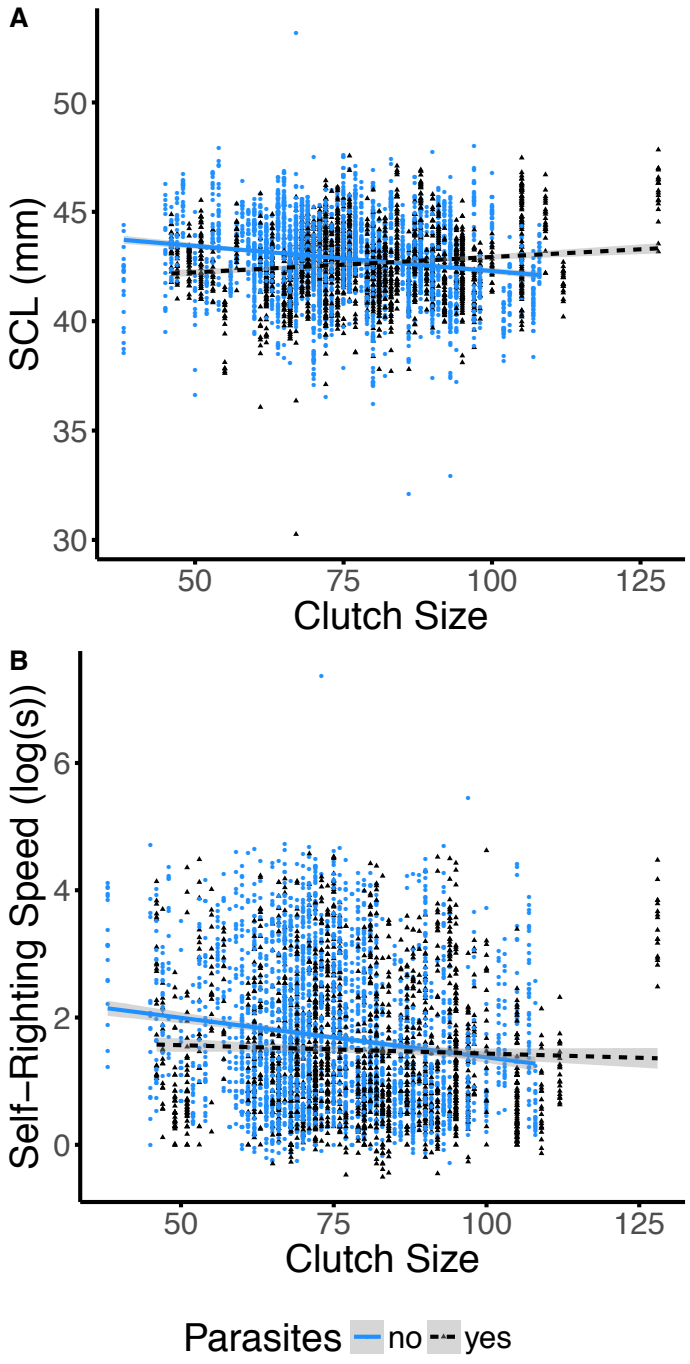

Figure A3.4: Scatterplots showing interaction between maternal infection status and clutch size had a significant association with both hatchling size (SCL: $F_{1,226} = 6.921$, $p = 0.009$) and self-righting speed ($F_{1,114} = 8.413$, $p = 0.004$). While plots are bivariate, statistics reported are from final reported multiple regression models.

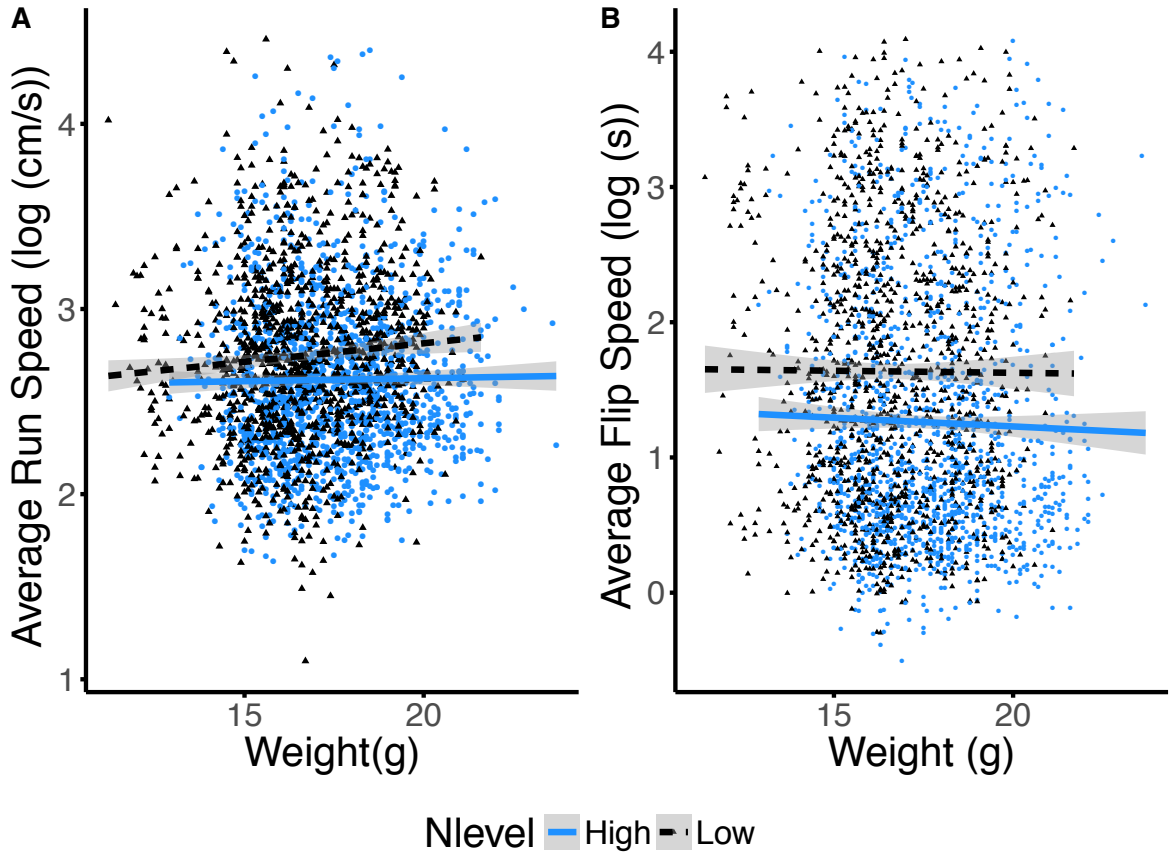

Figure A3.5: Scatterplots showing a significant interaction between the mass of a hatchling and the $\delta^{15}N$ of its mother on the speed it performs both crawl ($F_{1,2458} = 4.993$, $p = 0.026$) and self-righting tests ($F_{1,2525} = 5.163$, $p = 0.023$). Individuals from mothers that foraged at an enriched $\delta^{15}N$ level performed flip trials faster, but were slower in crawl tests. While plots are bivariate, statistics reported are from final reported multiple regression models.

Table A3.1: Composition of 20 µl PCR reactions for NADH and 18S rDNA. All PCR reactions were carried out under the same conditions: Thermo-cycling began with initial denaturation at 94 °C for 4 minutes followed by 45 cycles at 94 °C for 2 minutes 15 seconds, 44 °C for 20 seconds, 70 °C for 1 minute 30 seconds. The final extension lasted 7 minutes at 70 °C.

Constituent	**Volume**
Taq Polymerase (Biosystem Red Mix, 2x)	10 µl
F and R primer (5pmol/µl)	2 µl each
HPLC water	2 µl
template DNA	4 µl

Table A3.2: List of models tested. All two-way interactions between fixed effects were also included in full models.

Model	Response	Fixed Effects	Random Effects	Model Type
	Spatiotemporal trends			
1	Parasite Presence/Absence	Year, Island, CCL	NA	Generalised linear model (binomial)
2	Parasite Presence/Absence	Month, Island	Year	Generalised linear mixed effects model (binomial)
	Foraging Strategy			
3	δ15N	Parasite presence/absence, CCL	Island, Year	Linear mixed effects model
4	δ13C	Parasite presence/absence, CCL	Island, Year	Linear mixed effects model
	Reproductive Investment			
5	Average Egg Mass	Parasite presence/absence, CCL, δ15N, δ13C	Island, Year	Linear mixed effects model
6	Average Egg Size	Parasite presence/absence, CCL, δ15N, δ13C	Island, Year	Linear mixed effects model
7	Clutch Size	Parasite presence/absence, CCL, δ15N, δ13C	Island, Year	Linear mixed effects model
8	Clutch Mass	Parasite presence/absence, CCL, δ15N, δ13C	Island, Year	Linear mixed effects model
9	Success Rate	Parasite presence/absence, CCL, δ15N, δ13C	Island, Year	Linear mixed effects model (arcsine transformation)
	Trans-generational Effect			
10	SCL	Parasite presence/absence, CCL, Clutch Size, Incubation Duration, δ15N, δ13C	Nest, Island, Year	Linear mixed effects model
11	Mass	Parasite presence/absence, CCL, Clutch Size, Incubation Duration, δ15N, δ13C	Nest, Island, Year	Linear mixed effects model
12	Run Time	Parasite presence/absence, CCL, Clutch Size, Incubation Duration, δ15N, δ13C, hatchling mass	Nest, Island, Year	Linear mixed effects model
13	Flip Time	Parasite presence/absence, CCL, Clutch Size, Incubation Duration, δ15N, δ13C, hatchling mass	Nest, Island, Year	Linear mixed effects model
14	Flip Success Rate	Parasite presence/absence, CCL, Clutch Size, Incubation Duration, δ15N, δ13C, hatchling mass	Nest, Island, Year	Generalised linear mixed effects model (poisson)

Table A3.3: Statistical summary table reporting the best reduced models testing levels of infection over 1) year and 2) season. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

1) Infection across years	***d.f.***	**Chi-sq**	**p**
Year	1	194.669	**<0.0001**
Island	8	102.237	**<0.0001**
CCL	1	12.529	**<0.0001**
Year:Island	8	38.357	**<0.0001**
2) Infection across season			
Month	1	5.902	**0.015**
Island	8	63.722	**<0.0001**

Table A3.4: Statistical summary table reporting the best reduced models testing the effect of infection, CCL, $\delta^{15}N$ and $\delta^{13}C$, incubation duration, clutch size and hatchling mass, along with their two-way interactions, on offspring fitness tests including 1) Self-righting success 2) self righting speed and 3) crawl speed. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

1) Self-righting success	***d.f.***	**X^2**	**p**
Parasite Presence	1	0.526	0.468
CCL	1	2.2699	0.132
Incubation Duration	**1**	**4.77**	**0.029**
Clutch Size	1	1.312	0.252
Hatchling Mass	1	2.918	0.088
$\delta^{15}N$	1	0.007	0.935
Parasite Presence:CCL	**1**	**5.3002**	**0.021**
Parasite Presence:Clutch Size	1	3.681	0.055
CCL:Incubation Duration	**1**	**4.038**	**0.044**
CCL: $\delta^{15}N$	**1**	**7.327**	**0.007**
Incubation Duration:Clutch Size	1	3.435	0.064
2) Self-righting speed	***d.f.***	**F**	**p**
Parasite Presence	**1,115**	**7.087**	**0.009**
CCL	1,119	0.015	0.903
Incubation Duration	1,116	1.496	0.224
Clutch size	1,115	0.031	0.859
Hatchling Mass	**1, 2526**	**4.883**	**0.027**
$\delta^{15}N$	1,116	2.014	0.159
$\delta^{13}C$	1,113	0.582	0.447
Parasite Presence:CCL	1,119	2.298	0.132
Parasite Presence:Clutch Size	**1,114**	**8.413**	**0.004**
Parasite Presence: $\delta^{13}C$	1,114	3.819	0.053
CCL:Clutch Size	1,115	2.742	0.100
Incubation Duration:Clutch Size	**1,114**	**4.416**	**0.038**
Hatchling Mass: $\delta^{15}N$	**1, 2525**	**5.163**	**0.023**
Hatchling Mass: $\delta^{13}C$	**1, 2526**	**3.389**	**0.066**
3) Crawl Speed			
Parasite Presence	1,111	0.981	0.324
CCL	1,119	2.185	0.142
Incubation Duration	1,103	0.353	0.554

Clutch Size	1,110	0.011	0.918
Hatchling Mass	**1, 2458**	**5.003**	**0.025**
$\delta^{15}N$	1,75	3.295	0.073
$\delta^{13}C$	1,110	0.6799	0.411
Parasite Presence:Clutch Size	1,111	2.939	0.089
Parasite Presence: $\delta^{15}N$	1,112	2.688	0.104
Parasite Presence: $\delta^{13}C$	1,110	3.813	0.053
CCL:Clutch Size	1,110	2.308	0.131
CCL: $\delta^{15}N$	**1,167**	**8.323**	**0.004**
Incubation Duration:Clutch Size	**1,111**	**5.284**	**0.023**
Clutch Size: $\delta^{13}C$	1,110	2.4296	0.122
Hatchling Mass: $\delta^{15}N$	**1, 2458**	**4.993**	**0.026**
$\delta^{15}N$: $\delta^{13}C$	1,109	3.29	0.072

Appendix 4: Supplementary Material for Chapter 4

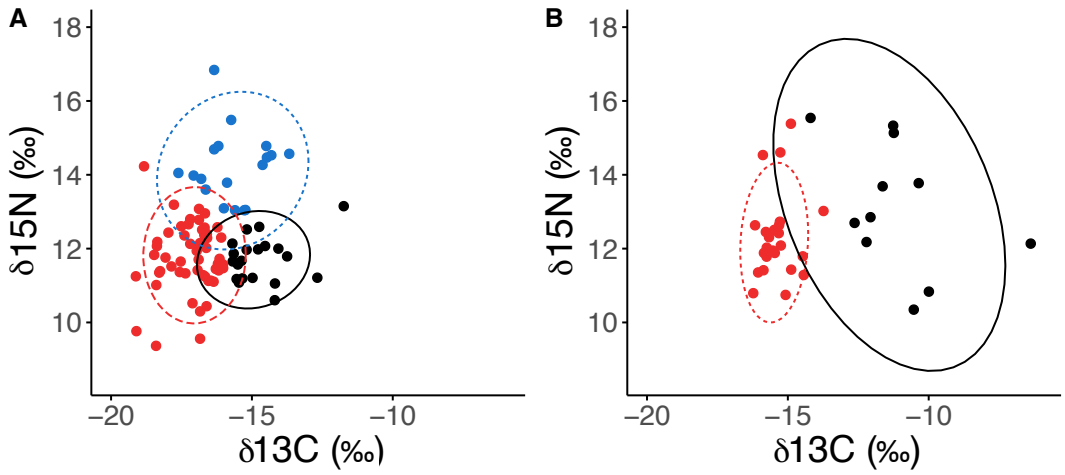

Figure A4.1: Scatterplots showing foraging strategies identified by AP clustering in A) 2016 and B) 2017. In 2016, three different feeding strategies were identified - one neritic strategy (black) and two oceanic, which were differentiated by a significant difference in d15N (red and blue). In 2017, two feeding strategies were identified - neritic (black) and oceanic (red).

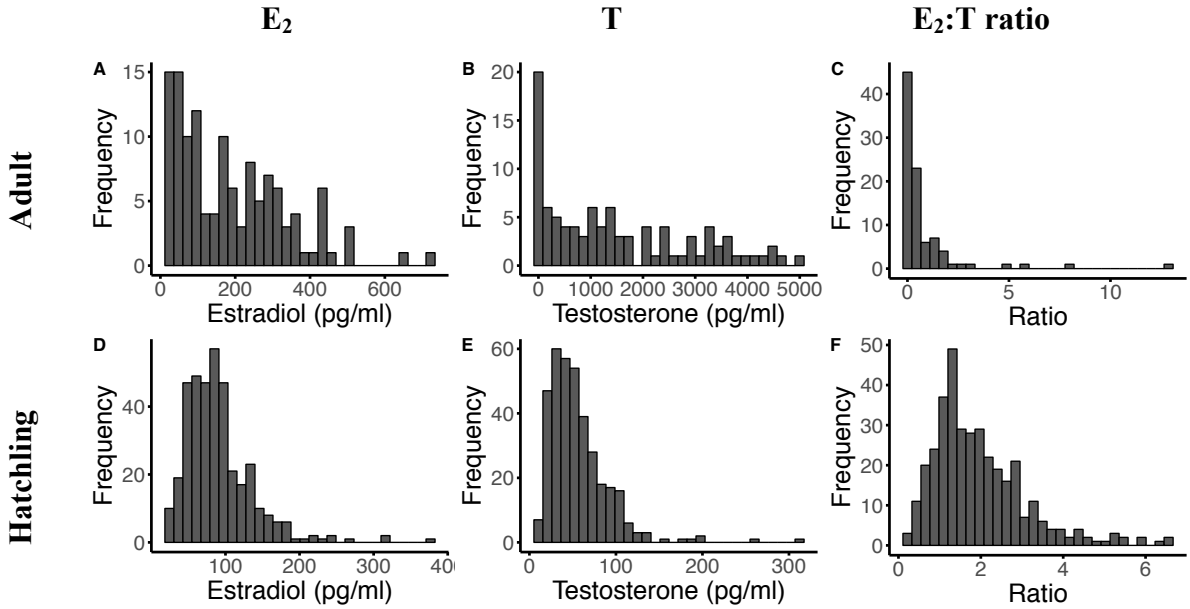

Figure A4.2: Histograms showing plasma hormone concentrations of nesting adult females and hatchlings show a positive skew and a high level of individual variation

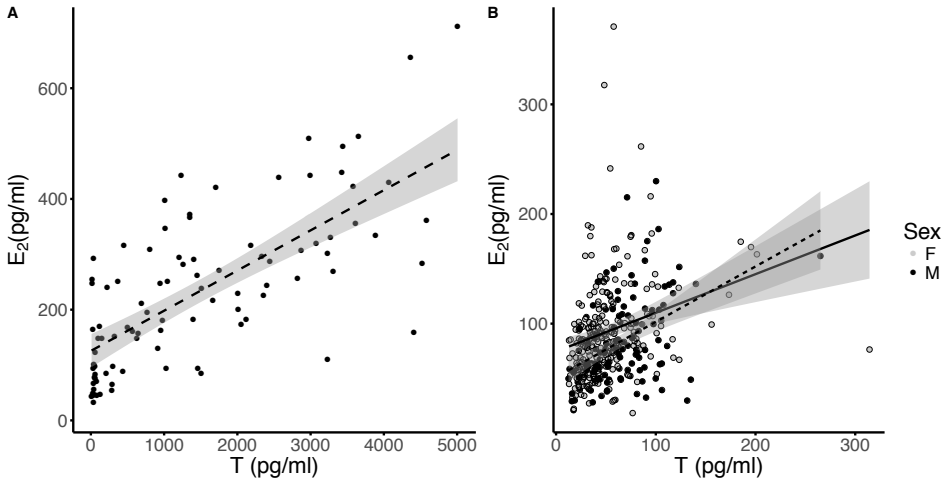

Figure A4.3: Scatterplots showing significant positive relationship exists between E_2 and T in the circulating plasma of A) adult nesting female turtles ($F_{1,91} = 74.909$, $p < 0.001$) and B) hatchlings, with the slope of increase being stronger in females ($F_{1,357} = 11.824$, $p < 0.001$).

Table A4.1: Description of foraging strategies as identified using $\delta^{15}N$ and $\delta^{13}C$ values and affinity propagation clustering.

				N		C		
	Cluster	**No. Indiv**	**% Indiv**	**Mean**	**SD**	**Mean**	**SD**	**Strategy**
2016	1	18	19.57	14.22	0.96	-15.68	1.07	Oceanic (upwelling)
	2	54	58.70	11.75	0.91	-17.16	0.84	Oceanic
	3	20	21.74	11.72	0.62	-14.71	1.05	Neritic
2017	1	12	32.43	13.56	2.21	-11.33	1.99	Neritic
	2	25	67.57	12.39	1.17	-15.37	0.59	Oceanic

Table A4.2: Statistical summary table reporting the best reduced models describing the relationships between individual environments and E_2, T and the E_2:T ratio in 2017. We included either strategy or **$\delta^{15}N$** and $\delta^{13}C$ as fixed variables, along with individual size and presence/absence of parasites. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

	E_2				***T***			***E_2:T***		
Strategy as a fixed variable		d.f	F	p		d.f	F	p	d.f	F	p
	CCL	1,20	0.811	0.378	Parasites	1,21	3.438	0.078	CCL	1,15	1.6904	0.213
	Parasites	1,20	2.991	0.099					Parasites	1,15	3.118	0.098
	Strategy	1,20	1.159	0.295					Strategy	1,15	2.248	0.154
	CCL:Strategy	1,20	3.465	0.077								
$\delta^{15}N$ and $\delta^{13}C$ as fixed variables	CCL	1,19	1.454	0.242	Parasites	1,16	2.841	0.111	CCL	1,12	2.146	0.169
	$\delta^{15}N$	1,19	0.989	0.332	$\delta^{15}N$	1,16	0.022	0.883	Parasites	1,12	3.434	0.089
	$\delta^{13}C$	1,19	2.131	0.161	$\delta^{13}C$	1,16	0.186	0.672	$\delta^{15}N$	1,12	1.247	0.286
	CCL: $\delta^{15}N$	1,19	1.051	0.318	N: $\delta^{13}C$	1,16	1.756	0.204	$\delta^{13}C$	1,12	1.666	0.221
	CCL: $\delta^{13}C$	**1,19**	**6.825**	**0.017**					CCL:Parasites	1,12	2.575	0.134
	$\delta^{15}N$: $\delta^{13}C$	1,19	5.685	0.028					CCL: $\delta^{15}N$	1,12	0.464	0.509
									N: $\delta^{13}C$	1,12	1.986	0.184

Table A4.3 Statistical summary table reporting the best reduced models describing the relationships between maternal phenotype and reproductive investment, in terms of clutch size and mass, egg size and clutch success. Fixed variables representing maternal phenotype included T and E_2 concentrations, CCL, infection status and $\delta^{13}C$ signature, along with all two-way interactions. All models were backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

	d.f.	**F**	**p**		**d.f.**	**F**	**p**
Clutch Size				*Egg Size*			
T	1,11	0.005	0.947	T	1,15	0.297	0.594
E_2	1,11	0.615	0.449	**CCL**	**1,15**	**26.225**	**< 0.001**
CCL	**1,11**	**6.270**	**0.029**	$\delta^{13}C$	1,15	0.043	0.838
$\delta^{13}C$	1,11	0.592	0.458	Year	1,15	0.009	0.925
Year	1,11	0.745	0.407	Parasites	1,15	1.008	0.331
Parasites	1,11	0.409	0.536	T:CCL	1,15	3.249	0.092
T: E_2	1,11	0.011	0.917	T: $\delta^{13}C$	1,15	0.122	0.732
T: $\delta^{13}C$	1,11	1.675	0.222	Year:E_2	1,15	1.599	0.235
T:Year	1,11	0.238	0.635	$\delta^{13}C$:Year	1,15	1.291	0.274
T:Parasites	1,11	0.465	0.509	$\delta^{13}C$:Parasites	1,15	1.223	0.286
Year:E_2	1,11	1.471	0.251				
Parasites:E_2	1,11	1.424	0.258				
CCL:Year	1,11	1.924	0.193				
CCL:Parasites	1,11	2.482	0.143				
Year:Parasites	**1,11**	**6.281**	**0.029**				
Clutch Mass				*Survival rate*			
T	1,9	4.332	0.067	T	1,8	0.58	0.468
E_2	1,9	1.535	0.247	E_2	1,8	0.917	0.366
CCL	**1,9**	**39.826**	**< 0.001**	CCL	1,8	2.024	0.193
$\delta^{13}C$	1,9	0.818	0.389	Parasites	1,8	0.145	0.714
Year	1,9	3.877	0.08	$\delta^{13}C$	1,8	1.503	0.255
Parasites	1,9	2.231	0.169	Year	1,8	0.075	0.791
T:E_2	1,9	0.001	0.974	T:CCL	1,8	0.509	0.496
T: $\delta^{13}C$	1,9	3.962	0.078	T:Parasites	1,8	0.511	0.495
T:Parasites	1,9	0.624	0.449	T: $\delta^{13}C$	1,8	3.173	0.113
E_2:CCL	1,9	0.551	0.477	T:Year	1,8	3.331	0.105
CCL: $\delta^{13}C$	1,9	2.314	0.163	E_2:CCL	1,8	2.154	0.18
CCL:Year	1,9	2.887	0.123	E_2:Parasites	1,8	0.91	0.368
CCL:Parasites	1,9	0.211	0.657	E_2: $\delta^{13}C$	1,8	1.618	0.239
$\delta^{13}C$:Year	1,9	3.293	0.102	E_2:Year	1,8	1.754	0.222
$\delta^{13}C$:Parasites	**1,9**	**6.307**	**0.033**	CCL:Year	1,8	0.369	0.56
				Parasites:Year	1,8	0.389	0.55
				$\delta^{13}C$:Year	1,8	3.048	0.119

Table A4.4: Reduced model describing the relationship between hatchling phenotype and self-righting speed. We included hatchling BMI and individual circulating T and E_2 levels as fixed effects with their two-way interactions as fixed effects, and also included clutch size and maternal strategy and infection status. Nest ID was included as a random effect. The model was backwards selected using AIC. Significant results highlighted in bold. D.f. denotes degrees of freedom.

	d.f	**F**	**p**
T	1,199	0.306	0.581
E_2	1,224	0.016	0.899
BMI	1,216	0.731	0.394
Temp	1,21	0.338	0.567
Parasites	1,21	0.057	0.813
$\delta^{13}C$	1,22	1.337	0.260