Assessing the resilience of sea turtle embryos to extreme temperatures

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Abstract

The survival and viability of sea turtle embryos is dependent upon favourable nest temperatures throughout the incubation period. Consequently, future generations of sea turtles may be at risk from increasing nest temperatures due to climate change, but little is known about how developing embryos will respond to thermal stress. Heat shock genes are likely to play an important role in resilience because they code for proteins that prevent cellular damage in response to environmental stressors. The experiments presented in this thesis provide the first evidence of sea turtle embryos using molecular mechanisms to mitigate the effects of acute thermal stress.

Sea turtle embryos (loggerhead, *Caretta caretta* and flatback, *Natator depressus*) were exposed to realistic and near-lethal temperatures of 36 °C for 3 hours. I investigated expression changes in the genes that code for heat shock proteins (*hsp*). Increased levels of expression of *hsp60*, *hsp70*, and *hsp90* mRNA in heart tissue were measured in response to a brief heat stress, and it was determined that both *hsp70* and *hsp90* were useful biomarkers for assessing heat stress in the late-stage embryos of sea turtles. This method can be used as a platform to investigate variation in the thermotolerance response from the clutch to population scale, which can help anticipate the resilience of reptile embryos to extreme heating events.

To estimate whether expression of *hsp* is heritable in sea turtle embryos, I had to better understand the underlying level of genetic variation that could contribute to thermotolerance. As female sea turtles are promiscuous, multiple sires often contribute to a clutch of eggs, but rates of multiple paternity can vary greatly within and across species. I investigated levels of multiple paternity in *C. caretta* from three rookeries in Western Australia by analysing polymorphic species-specific genetic markers. I predicted that level of multiple paternity would be related to nesting population size and hence the large rookery at Dirk Hartog Island would have higher rates of multiple paternity than the two smaller mainland rookeries at Gnaraloo Bay and Bungelup Beach. Contrary to my prediction, I found highly variable rates of multiple paternity among the
rookeries sampled, which was unrelated to female population size (25 % at Bungelup Beach, 86 % at Gnaraloo Bay, and 36 % at Dirk Hartog Island). Approximately 45 different males sired 25 clutches and the average number of sires per clutch ranged from 1.2 to 2.1, depending on the rookery sampled. The variance in rates of multiple paternity among rookeries suggests that operational sex ratios are variable in Western Australia. Periodic monitoring would show if the observed patterns of multiple paternity for these three rookeries are stable over time, and my data provide a baseline for detecting shifts in operational sex ratios.

From earlier experiments it was confirmed that genes coding for hsp60, hsp70, and hsp90 increase expression under heat stress to maintain tissue viability. If species are to adapt to climate change, the expression levels of these genes must be heritable. This is especially important for egg-laying sea turtles, as they exhibit extreme fidelity to nesting sites and have narrow tolerances of incubation temperatures. The regions in which sea turtle embryos develop vary by population and species, with nesting beaches confined mostly to the temperate and tropical zones.

In C. caretta, there was significant geographic variation in the expression of hsp90 between a temperate and a sub-tropical rookery, but it was unclear if this was due to maternal effects (genetic factors) or environmental effects (non-genetic factors). Both maternal and paternal genetic variance (narrow-sense heritability) contributed to the expression of hsp60 and hsp70, while environmental factors more than genetic factors (broad-sense heritability) contributed to the response of hsp90. Additionally, there was a positive relationship between baseline levels of expression and increased levels of expression for hsp60, hsp70, and hsp90. These correlations suggest that if a gene expressed highly in one thermal environment it would also increase expression in another. Further to this, there were positive genetic correlations between hsp60 and hsp70 in the heat shock treatment. This suggests the increased expression of one gene influences the increased expression of the other gene, and thus both genes may be necessary for resilience to thermal stress.
Contrary to *C. caretta*, geographic variation in the expression of *hsp* was not detected between an island and mainland rookery for *N. depressus*. Neither the effect of rookery or clutch contributed to the variable expression of all target genes. Additionally, the heritability of *hsp* expression was not assessed in *N. depressus* due to financial and time constraints. However, expression profiles of all *hsp*s and the variance in this expression were compared between *N. depressus* and *C. caretta*. In the between-species comparison, clutch contributed the most significant proportion of variation in all target genes. Despite the genetic differences between the species, neither species nor rookery significantly contributed to the variation in the expression of all *hsp*s. The most likely explanation for lack of interspecific variation is statistical power for this experiment was low and therefore results should be interpreted with caution.

Higher incubation temperatures are likely given the rapid pace of environmental warming, and as a consequence higher temperatures may have profound impacts on development and hatching success of sea turtle embryos. However, plasticity in expression of heat shock genes may offer some resilience to thermal and other stressors and provide a platform for adaptation to warmer climates through selection on these genes. But, the limitations of plasticity in expression of heat shock genes requires further study given the current pace of environmental change.
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Declarations and Publications List

All parts of this thesis were written by Jamie Tedeschi, in consultation with Nicola Mitchell (Co-ordinating Supervisor) and Jason Kennington (Co-Supervisor) from UWA, Scott Whiting (Co-Supervisor) from Western Australian Department of Parks and Wildlife (DPaW), Oliver Berry (Co-Supervisor) from Commonwealth Scientific and Industrial Research Organisation (CSIRO), and Mark Meekan (Co-Supervisor) from Australian Institute of Marine Science (AIMS). Chapter 4 was also written in consultation with Joseph Tomkins from UWA. This thesis is presented as a combination of scientific papers and thesis chapters. Below are estimates of my contributions to each chapter.

Chapter 1: General Introduction

- Writing: JN Tedeschi 95%


- Concept: 20%
- Planning: 30%
- Data Collection: 70% (20% field, 50% laboratory)
- Analysis: 60%
- Writing: 75%

- Concept: 40%
- Planning: 40%
- Data Collection: 70% (20% field, 50% laboratory)
- Analysis: 80%
- Writing: 75%

**Chapter 4:** submitted to *Proceedings of the Royal Society B: Biological Sciences* as Tedeschi JN, Kennington WJ, Tomkins JL, Berry O, Whiting S, Meekan MG, & Mitchell NJ.

- Concept: 20%
- Planning: 40%
- Data Collection: 70% (20% field, 50% laboratory)
- Analysis: 50%
- Writing: 65%

**Chapter 5:** not yet published. To be submitted under authorship Tedeschi JN, Mitchell NJ, Tomkins, JL, Berry O, Whiting S, Meekan M, & Kennington WJ.

- Concept: 30%
- Planning: 40%
- Data Collection 70% (20% field, 50% laboratory)
- Analysis: 80%
- Writing: JN Tedeschi 95%

**Chapter 6: General Discussion**

- Writing: JN Tedeschi 95%
The work associated with the production of these papers/chapters in this thesis is my own. The contribution of the different co-authors in the papers/chapters is mainly associated with the initial research directions, advice on experiments and data analysis when required, and editorial input in various versions of the drafts on the papers/chapters. The photographs on the Title Page were taken by Jamie Tedeschi and Kasey Darts (Western Australian Department of Parks and Wildlife).

____________________________

Jamie Tedeschi

May 2015
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Chapter 1: General Introduction

Industrialization since the 19th century has triggered a steep rise in CO₂ emissions that do not readily dissipate within the atmosphere (IPCC 2014). Over the second half of the 21st century, these emissions are expected to increase global mean temperatures by 3.5 °C (under low-emission scenarios) to over 11.0 °C (under high-emission scenarios) compared to ‘recent’ mean global temperatures (1986-2005) (IPCC 2014). Extreme changes in climate are a major threat to biodiversity (Bellard et al. 2012) by negatively impacting species distributions, phenology, behaviour, reproduction, and physiology (Gitay et al. 2002; Walther et al. 2002; Root et al. 2003; Maclean & Wilson 2011). Some species have been forced to shift distributions to track optimal climate niches (Parmesan 2006; Chen et al. 2011), but those species that are poor distributors or cannot cross natural or human barriers (Schloss et al. 2012) face high risks of extinction (Parmesan 2006; Schloss et al. 2012). Climate from the 20th century has already caused shifts in key phenological events across several taxa; flowering has advanced by more than 10 days per decade in some species (Parmesan 2006). Physiological responses may be in the form of tolerating warmer or drier climates and modifying diets, energy budgets, and activity levels (Bellard et al. 2012). Ectotherms are particularly sensitive to environmental changes (Bellard et al. 2012), as increasing temperatures can
negatively impact locomotion, growth, reproduction, and sex determination (Tewksbury et al. 2008). However, phenotypic plasticity of these responses and genetic adaptation could allow such species to persist in their current environments despite climate change (Urban et al. 2014). Temperature increases will likely reduce fitness in some populations and thus could cause strong selection on climate-related traits (Urban et al. 2014).

Climate warming is likely to have a negative impact on sea turtles, because as ectotherms their life history traits, behaviour, and physiology are strongly influenced by environmental temperature (Spotila & Standora, 1985; Janzen, 1994). Sand temperature during egg incubation plays a vital role in embryo development, hatching sex ratio, and hatching success (Davenport 1997; Ackerman & Lott 2005), thus increases in temperature may alter hatching attributes and survival (Booth & Freeman, 2006). Therefore, sea turtles are an interesting ‘canary in the coal mine’ species to study the effects of climate change in marine habitats (Hawkes et al. 2014).

1.1 Sea turtle life history

The seven extant species of sea turtles differ in many aspects of their ecologies, but they are extremely similar in being long-lived and their basic life histories (Pritchard 1997; Plotkin 2002). Life-cycle characteristics are similar in all sea turtle species, with a breeding migration from foraging areas to mating and nesting areas (Fig. 1.1.1) (Lanyon et al. 1989). During the breeding period, males and females migrate to mating areas close to the nesting beaches (Godley et al. 2002a; Hays et al. 2010). Following mating, the males return to their foraging grounds and the females spend several months at the nesting area laying multiple clutches (Godley et al. 2002a; Hays et al. 2010). During a breeding cycle, females come ashore approximately every two weeks, and lay up to seven clutches throughout the nesting season (Balazs 1981; but see Bjorndal & Zug 1995; Davenport 1997). Female sea turtles return to their natal beaches every 1-4 years once they become sexually mature (Balazs 1981; Lohmann et al. 2013), and genetic studies have demonstrated that all species
are polygynous and polyandrous to varying degrees (Harry & Briscoe 1988; Kichler et al. 1999; Lee & Hays 2004; Stewart & Dutton 2011).

Hatching is temperature-dependent and emergence from nests occurs after a 6-13 week incubation period (Miller 1997). Hatchlings generally emerge onto the beach at night when sand temperature is coolest and follow the horizon toward the sea to begin their developmental migration (Lohmann et al. 1997). Hatchlings leave their natal beaches and migrate to deep-water oceanic nursery habitats where they spend 5-20 years as non-reproductive juveniles (Musick & Limpus 1997). Juvenile turtles recruit to shallow near shore feeding grounds until they reach sexual maturity (Musick & Limpus 1997). Finally, the mature adult turtles then migrate to the general vicinity of their natal beaches to begin the reproductive cycle again (Miller 1997).

Fig. 1.1.1 A generalised life cycle of sea turtles, from Lanyon et al. (1989).
1.2 Thermal biology of sea turtle embryos

Sea turtles construct deep nests compared to other oviparous reptiles, with the depth of the narrow nest chambers primarily dependent on the length of the female’s hind flippers. Eggs settle at a range of depths during oviposition, and a thermal gradient develops between the top and bottom layers of eggs (Booth & Astill 2001a; Ackerman & Lott 2005). This gradient, together with egg size, clutch size, and the metabolic heat generated by hatchlings later in development, may raise the overall nest temperature well above that of the surrounding sand (Spotila et al. 1987; Mrosovsky 1994; Godfrey et al. 1997; Booth & Astill 2001b).

Hatchling success is closely tied to incubation temperatures, with low hatching success at temperatures outside of the range of 25-35 °C (Davenport 1997, Miller 1997). Balanced offspring sex ratios are also vulnerable to increasing nest temperatures. During the middle third of development, a pivotal temperature of ~29 to 31 °C (Standora & Spotila 1985; Wibbels 2003) produces a 1:1 ratio of males:females (depending on species and population). Altering the temperature by 0.5 °C either side of the pivotal value can change the sex ratio dramatically (Hewavisenthi & Parmenter 2002), with more males produced at temperatures below 29°C, and the possibility of complete feminisation of clutches at temperatures above the pivotal temperature (Hawkes et al. 2007; Laloë et al. 2014).

Embryonic development continues at temperatures near the upper critical limit (~33 °C according to Ackerman 1997 and ~35 °C according to Miller 1997), but moisture is absorbed at a faster rate, producing smaller offspring with larger residual yolk (Packard et al. 1987; Davenport 1997; Packard 1999; Deeming 2005; Booth & Evans 2011). Furthermore, nest temperatures above 35 °C during the final weeks of incubation can increase hatchling mortality for several reasons: 1) development can be arrested at various stages of growth, 2) hatchlings can pip out of the shell, but may be unable to breach the surface of the nest, and 3) hatchlings breaching the surface of the nest may succumb to heat exhaustion before making it to sea (Blamires & Guinea 2001; Drake & Spotila 2002; Valverde et al. 2010; Telemeco et al. 2013a; Pike 2014). Extreme
heat events can reduce clutch hatch success or even result in complete clutch failure (Matsuzawa et al. 2002; Hawkes et al. 2007; Maulany et al. 2012) making some nesting areas unsuitable for hatchling development during some parts of the year (Hawkes et al. 2007).

1.3 Potential for sea turtles to adapt to climate change

1.3.1 Behavioural mechanisms to moderate thermal stress

How sea turtles moderate the negative effects of climate change will depend on their ability to adapt to increasing temperatures (Howard et al. 2014). Modes of adaptation include changes in nesting phenology (Weishampel et al. 2004; Pike et al. 2006; Telemeco et al. 2013b), changes in nest-site choice on natal beaches (Weber et al. 2012), changes in nesting location along a latitudinal gradient (Hawkes et al. 2007; Pike 2014), and possibly (but somewhat limited) behavioural thermoregulation by embryos inside the egg (Du et al. 2011; Zhao et al. 2013).

However, the pace of contemporary climate change may be quicker than the pace at which sea turtles can behaviourally adapt (Hamann et al. 2013). For example, the onset of breeding is often triggered by increase of water temperatures at foraging grounds (Hays et al. 2002; Mazaris et al. 2004), but sea turtles may have to travel hundreds to thousands of kilometres between foraging and nesting grounds (Carr 1975). There may be a lag in response because the locations of breeding initiation and nesting differ; therefore, it could take generations for populations to shift timing of breeding (Hamann et al. 2007). Although there is recent evidence of local adaption of some populations to warmer incubation temperatures (Weber et al. 2012), the rate of climate change may outpace nesting sea turtles ability to select for beaches that may provide cooler temperatures (e.g. sand colour, grain size, aspect, vegetation cover, etc.; Booth & Freeman 2006; Poloczanska et al. 2009). Lastly, even though embryos are fixed in their environments, they are able to shift position inside the egg to seek optimal temperatures, although only in the earliest stages of development (Du et al. 2011; Zhao et al. 2013).
1.3.2 *Physiological mechanisms to moderate thermal stress*

Embryos have limited behavioural means to manipulate their incubation environment to maintain favourable temperatures during development (Due et al.; Zhao et al. 2013). Therefore, it is possible they could have a greater reliance on physiological means by which to mitigate the effects of increasing incubation temperatures and subsequent thermal stress (Gao et al. 2014). The cellular stress response begins at the molecular level with the activation of stress proteins, such as heat shock proteins (Hsps) (Kültz 2005). Hsps are found across Kingdoms and are highly conserved, with low variation in the coding regions between species (reviewed by: Feder & Hofmann 1999; Kültz 2005; Sorensen et al. 2003). Both the low variation and presence of heat-shock proteins in all living organisms suggest they play an important role in protecting cells during or after stress (Feder & Hofmann 1999; Sorensen et al. 2003).

Hsp70 is the best described Hsp family and in many organisms consists of combinations of inducible (activated under stressful conditions) and constitutive (baseline expression under normal conditions) forms (Angilletta et al. 2002; Kültz 2005). Many studies on critical thermal maxima of ectotherms have provided evidence of the capability of species to increase expression of Hsps or genes that code for Hsps (*hsp*s) in response to heat stress. A growing proportion of this work has been able to determine some fitness costs associated with environmental stress. For example, Ramaglia & Buck (2004) and Stecyk et al. (2012) have shown adult fresh water turtles are able to change expression levels of Hsp60, Hsp70, and Hsp90 in response to changes in temperatures, and Gao et al. (2014) detected overexpression of *hsp70* in embryonic Chinese soft-shelled turtles (*P. sinensis*). These studies were the building blocks of my PhD project – determining the genetic and molecular basis of thermotolerance in sea turtle embryos.

When exposed to stressful conditions, individuals have three possible responses: (1) avoid the stress, either by moving to a more favourable habitat, adjusting activity patterns or changing to a physiological state that might be more resistant (e.g. hibernation or diapause); (2) adapt to the stressful condition by selection or by individuals responding plastically; or (3) fail in the above and
go extinct (Skelly et al. 2007; Hoffmann & Sgrò 2011; Bellard et al. 2012). The experiments designed in this thesis focus on the second option: adaptation through plasticity in expression of heat shock protein genes, or selection acting on those genes. The potential for increased expression of heat shock genes as a mechanism for adaptation to environmental stress is not known for sea turtles, but changes in gene expression levels of \textit{hsp60} and \textit{hsp70} have been detected in fresh water turtles in anoxic and thermally stressful environments (Ramaglia & Buck 2004; Stecyk et al. 2012; Gao et al. 2014). As heat shock proteins influence fitness under sub-optimal environmental conditions, it can be argued that the regulation and expression levels of these genes and proteins are of major evolutionary and ecological importance (Feder & Hofmann 1999; Kültz 2005; Sorensen et al. 2003).

As turtle embryos have limited capacity to thermoregulate by behavioural mechanisms (Du et al. 2011; Zhao et al. 2013), I hypothesised that sea turtle embryos rely on physiological mechanisms to mitigate potential deleterious consequences of high incubation temperatures during development, and that this response is detectable when embryos are exposed to brief periods of heat stress (e.g. by increasing expression of heat-sensitive genes such as heat shock proteins). But, we also need to know about the genetic basis of trait variation and extent of phenotypic plasticity, so I hypothesised that there will be heritable variation in the expression of heat shock genes since adaptation of thermotolerance requires a platform for which selection can act.

1.4 Gaps in knowledge

A large number of studies on the impacts of climate change on sea turtles have focused on changes in nesting phenology (Weishampel et al. 2004; Pike et al. 2006; Telemeco et al. 2013b), offspring sex ratio bias (Godley et al. 2002b; Wibbels 2003; Hawkes et al. 2007; Hays et al. 2010; Katselidis et al. 2012; Laloë et al. 2014), hatching success Blamires & Guinea 2001; Drake & Spotila 2002; Valverde et al. 2010; Telemeco et al. 2013a; Pike 2014), and the subsequent decline of global population numbers (Limpus 2008; Schwanz & Janzen 2008; Kallimanis 2010; Fuentes et al. 2011). Many researchers have
investigated the potential for the adaptive value of TSD, (Hawkes et al. 2007; Mitchell & Janzen 2010) or else quantified pivotal temperatures in order to be able to predict sex ratios of hatchlings (Hewavisenti & Parmenter 2002; Hawkes et al. 2007; Laloë et al. 2014; Stubbs et al. 2014). However, to fully understand the implications of climate change on the development of embryos, studies need to quantify resilience of populations (e.g. plasticity of thermotolerance) (Fuentes et al. 2013) and to estimate evolutionary responses to heat stress (reviewed by Hansen et al. 2012 and Howard et al. 2014). Whilst field observations on overheating and hatchling failure in turtles exist (Blamires & Guinea 2001; Drake & Spotila 2002; Escobedo-Galván et al. 2011), relatively few studies have looked experimentally at thermal tolerance of embryos in sea turtles. The lack of accurate quantitative information limits the capacity to manage potential climate change effects on turtles, or evaluate the risks they face.

1.5 Research objectives

Most empirical studies on sea turtle embryos have investigated the role of incubation temperature on sex determination and the possibility of female-biased primary sex ratios (Yntema 1968; Miller 1985; Mrosovsky 1994; Davenport 1997; Godfrey et al. 1997; Miller et al. 2003; Birchard 2005), but have not considered the more fundamental question of the thermal influence on trait variation and fitness.

The overall goal of this thesis is to quantify phenotypic variation in heat shock gene expression in two of the seven extant species of sea turtles, to assess the plasticity of the heat shock response, and to determine whether this response is heritable.

I had the following specific aims:

1. To design an incubation protocol to induce a heat shock response in sea turtle embryos, and quantify that response with gene expression assays for hsp60, hsp70, and hsp90 (Chapter 2).
2. To conduct paternity analysis to estimate the level of genetic variation within clutches for the third largest nesting population of loggerhead turtles (Chapter 3).

3. To quantify the phenotypic variation in heat shock gene expression in response to heat stress in loggerhead turtles - between clutches within populations, and between populations within species (Chapter 4).

4. To use paternity information (Chapter 2) to estimate the heritability of the heat shock response in loggerhead turtle embryos (Chapter 4).

5. To quantify the phenotypic variation in heat shock gene expression in response to heat stress in flatback turtles (as was done for loggerhead turtles) and thereby to compare *hsps* expression profiles in two species (Chapter 5).

1.6 Thesis outline

This thesis is in accordance with the Postgraduate and Research Scholarship Regulation 1.3.1.33(1) of the University of Western Australia, Australia and is presented as a series of scientific papers. Two papers have been published, and one other paper has been submitted for publication. There are six chapters in this thesis, consisting of a general introduction, four data chapters and a general discussion. Experimental chapters can be read either as a part of the whole thesis or as separate entities. Each data chapter contains an Abstract, Introduction, Materials and Methods, Results, Discussion and References, and there is some unavoidable overlap, especially in the Materials and Methods sections of Chapters 2, 4 and 5, and in the Results sections of Chapters 4 and 5. The general discussion evaluates the important issues raised by the research reported in this thesis, draws together key conclusions, and highlights areas for further research. A breakdown of author contributions to each chapter are presented on a separate declaration page.
1.7 References


Maulany R, Booth D, Baxter G. 2012 Emergence success and sex ratio of natural and relocated nests of olive ridley turtles from Alas Purwo National


Schloss CA, Nunez TA, Lawler JJ. 2012 Dispersal will limit ability of mammals to track climate change in the Western Hemisphere. *PNAS* 109, 8606–8611. (doi:10.1073/pnas.1116791109)


Chapter 2: Increased expression of *Hsp70* and *Hsp90* mRNA as biomarkers of thermal stress in loggerhead turtle embryos (*Caretta caretta*).

2.1 Abstract

The survival and viability of sea turtle embryos is dependent upon favourable nest temperatures throughout the incubation period. Consequently, future generations of sea turtles may be at risk from increasing nest temperatures due to climate change, but little is known about how embryos respond to heat stress. Heat shock genes are likely to be important in this process because they...
code for proteins that prevent cellular damage in response to environmental stressors. This study provides the first evidence of an expression response in the heat shock genes of embryos of loggerhead sea turtles (*Caretta caretta*) exposed to realistic and near-lethal temperatures (34 °C and 36 °C) for 1 or 3 hours. We investigated changes in *Heat shock protein 60* (*Hsp60*), *Hsp70*, and *Hsp90* mRNA in heart (*n* = 24) and brain tissue (*n* = 29) in response to heat stress. Under the most extreme treatment (36 °C, 3 h), *Hsp70* increased mRNA expression by a factor of 38.8 in heart tissue and 15.7 in brain tissue, while *Hsp90* mRNA expression increased by a factor of 98.3 in heart tissue and 14.7 in brain tissue. Hence, both *Hsp70* and *Hsp90* are useful biomarkers for assessing heat stress in the late-stage embryos of sea turtles. The method we developed can be used as a platform for future studies on variation in the thermostolerance response from the clutch to population scale, and can help us anticipate the resilience of reptile embryos to extreme heating events.

### 2.2 Introduction

The earth is undergoing rapid warming due to climate change, a process driven by the release of carbon from anthropogenic sources into the atmosphere (IPCC, 2007). In recent years, awareness of this issue has led to many studies reporting the physiological and underlying genetic response to heat stress in a wide range of organisms. For ectotherms, this response varies greatly by taxa, e.g. insects (Bahrndorff et al., 2010; Hoffmann et al., 2003; Krebs et al., 1998), fish (Currie et al., 2000; Fangue et al., 2006; Meffe et al., 1995), frogs (Sorensen et al., 2009), lizards (Ulmasov et al., 1992), and fresh water turtles (Chang et al., 2000; Du et al., 2011; Li et al., 2012; Prentice et al., 2004; Ramaglia and Buck, 2004; Stecyk et al., 2012).

At the most basic level, an organism’s response to increasing temperatures is molecular (Kenkel et al., 2014) beginning with the activation of response genes (expression), including the increase in production of molecular chaperones and specialised enzymes to mitigate damage to membranes, proteins, and genomic material (Kultz, 2005). Because they are highly conserved across most taxa (Feder and Hofmann, 1999; Kultz, 2005; Rupik et al., 2011; Sorensen et al., 2012).
heat shock proteins (hsp) have been a particular focus of research. Under heat stress conditions, hsp are capable of increasing expression in response to the stressor (Evans and Hofmann, 2012; Feder and Hofmann, 1999; Kenkel et al., 2014;) and can alter an organism’s thermal sensitivity (Angilletta, 2009; Kultz, 2005).

Protein stability and folding is maintained by some of the larger kDa proteins, specifically the Hsp60 and Hsp70 families (Feder and Hofmann, 1999; Martin et al., 1992). Hsp70 is also responsible for translocating proteins across cellular membranes and protecting neurons from apoptosis (Feder and Hofmann, 1999; Gething and Sambrook, 1992; Mailhos et al., 1994). The Hsp90 family acts as chaperones for steroid and hormone receptors, participates in cell signalling and provides myelination for protecting neuronal cells (Pratt, 1998; Mailhos et al, 1994; Wyatt et al., 1996). These protein families can increase expression in heart and brain tissue when exposed to high temperatures (Hutter et al., 1994; Krivoruchko and Storey, 2010) and preserve the functioning of tissues until the stress period is relieved (Feder and Hofmann, 1999; Kultz et al., 2005; Rupik et al., 2011).

Sea turtle embryos develop optimally within a narrow thermal range, and are vulnerable to mortality if temperatures exceed 35 °C (Ackerman, 1997; Davenport, 1997). Average incubation temperatures above 35 °C early in development rapidly increase the rate of metabolic activity and development, resulting in small embryos that hatch early (Davenport, 1997; Deeming, 2005). Temperatures above 35 °C late in development can reduce the fitness of hatchlings by altering swimming performance, causing disorientation and death from heat exhaustion (Blamires and Guinea, 2001; Drake and Spotila, 2002; Valverde et al., 2010). Climate change is expected to increase air temperatures by 3.5 °C in the coming decades (IPCC, 2007) and this will in turn increase the temperatures of nesting beaches. Little is known about the capacity of embryonic turtles to tolerate and adapt to warming nest environments (Fuentes et al., 2009; Weber et al., 2011). Thus, the physiological and molecular mechanisms underlying the thermotolerance response of sea turtle embryos need to be investigated (Hawkes et al., 2014; Pike, 2014).
Loggerhead sea turtles (*Caretta caretta*, Linnaeus 1758) are globally distributed (Baldwin et al., 2003) but have a relatively narrow nesting range compared to other sea turtles, mostly confined to the sub-tropics where beaches may experience significant variation in temperature during the nesting season (Pike, 2014). One of the world’s largest loggerhead rookeries occurs on Dirk Hartog Island in Shark Bay, Western Australia (Baldwin et al., 2003; Reinhold and Whiting, 2014). Eggs buried on these beaches experience temperatures upwards of 35 °C (Woolgar et al., 2013) and occasional lethal heating events (Trocini, 2013) that are likely to increase in frequency as air temperatures rise under climate change. Here, through artificial incubation, we simulate *in situ* thermal stress and measure changes in mRNA levels of *Hsp* gene expression in response to that stress in *C. caretta* embryos. We hypothesise that the thermally sensitive *Hsp60*, *Hsp70*, and *Hsp90* genes will increase mRNA expression in embryos as a response to heat stress, but more specifically that these genes will be good indicators of a thermotolerance response in this species. To test our hypothesis, we first developed a protocol designed to elicit and quantify a thermal response in embryos. Additionally, we compared the responses in whole heart and whole brain tissue, as these differ in heat-shock protein abundance and potentially have different gene expression profiles (Stecyk et al., 2012). Further, we assessed the impact of brief heat shocks on hatching success to determine whether our treatments were lethal, sub-lethal or benign. Finally, we discuss our findings as they relate to previous studies investigating thermotolerance responses in reptiles, as they are the first reported for the embryonic stage in the Cheloniidae lineage.

### 2.3 Materials and Methods

#### 2.3.1 Animals
This study was approved by the University of Western Australia Animal Ethics Committee (RA/100/3/1081) and conforms to relevant guidelines for the care of experimental animals. Euthanasia protocols follow the American Veterinary
Medical Association guidelines for embryonic reptiles (Conroy et al., 2009; Leary et al., 2013).

2.3.2 Egg Collection
Loggerhead turtle eggs were collected from Turtle Bay on Dirk Hartog Island in Western Australia (25°29’59.47” S, 112°59’35.90” E) in January 2012. Ninety-six eggs were collected from each of four clutches (total eggs = 384). They were excavated from the nest chamber after the female returned to the water, and were individually numbered, and packed in chilled sand for transportation off the beach. Within three hours of collection, eggs were re-packed in moist vermiculite in a 40 L portable refrigerator (ENGEL MT45F-S, Australia) set at 7–10 °C to suspend embryonic development (Harry and Limpus, 1989). Finally, chilled eggs were transported by boat and road to the University of Western Australia in Perth for incubation within a 72-hours of oviposition.

2.3.3 Incubation and monitoring
Eggs were gradually warmed to room temperature in the laboratory and distributed among 1.5 L plastic containers half filled with moist sand, producing 96 containers of 4 eggs, each containing one egg from each female. Two 500 L incubators (Steridium, Model e500, Brisbane, QLD, Australia) were set at the pivotal temperature of 29.0 °C for sex determination in this population (Woolgar et al., 2013). Pivotal temperatures are viewed as optimal temperatures for embryonic development and viability (Ackerman and Lott, 2005; Drake and Spotila, 2002). Sand was sprayed with de-ionized water every 2 d to maintain humidity, and containers were systematically moved between shelves and incubators to minimise any effects of subtle temperature variation within and between the incubators.

Day 0 of incubation was the start point for calculating the time to each treatment, and to hatching. Loggerhead embryos are defined as being in the early stages of incubation from day 0 to day 25 and in the late stage of incubation from approximately day 45 up until hatching at around 58 days (Miller, 1985; Miller et al., 2003; Mrosovsky, 1980; Yntema, 1968). All containers were incubated at an average of 29.3 °C (± 0.3 °C) until day 25 or
day 45 of incubation when they received a heat shock, except for two procedural control groups that remained at 29 °C. Embryos that were not sacrificed after the heat shock treatments were returned to their original incubator and incubated until hatching (Fig 2.1).

![Factorial block design diagram](image)

**Figure 2.1.** A Factorial block design, sample sizes for each procedural control (PC1 and PC2) and heat shock treatment (T1, T2, T3, T4) at either Day 25 or 45 of incubation. Subsets of embryos were euthanised or left to hatch. Eggs were divided so that 4 replicates from each of the 4 clutches were in each treatment (treatment n = 64; total n = 384).

### 2.3.4 Heat Shock Treatments

Two procedural control (PC1 and PC2) and four heat shock treatments (T1, T2, T3, and T4) were designed to determine the best combination of parameters for inducing a response to thermal stress (Fig 2.1). These treatments were chosen because the temperatures were 1 °C on either side of the lethal field
temperature reported by Davenport (1997), and because shocks of 1 or 3 hours simulate realistic environmental fluctuations without inducing instant mortality. The temperature and duration of the shock that the embryos experienced was divided into two blocks of experiments (Fig 2.1). Egg containers received heat shocks at either Day 25 or Day 45 of incubation (termed Day 25 or Day 45 embryos throughout). Eggs were further allocated to either be euthanised immediately, or left to hatch and hence were returned to the 29 °C incubator (approximately 50 %) (Fig 2.1). We predicted that the most extreme heat shock, treatment 4 (36 °C, 3 h), would create a thermally stressful environment for embryos and gene expression levels would greatly increase in response.

We determined the time taken for the ‘heat shock’ to be detected within the incubation medium using small temperature loggers (Thermocron® iButton DS1921H, Thermodata, Brisbane, QLD, Australia; accuracy ±0.125 °C, precision, ±0.125 °C) buried amongst the eggs. Temperatures reached the set points of 34 °C and 36 °C after two hours. Thus, containers remained inside the incubators at the heat shock temperature for a total of three hours (2 h ramping + 1 h shock) or five hours (2 h ramping + 3 h shock), depending on the treatment group. The procedural control groups were moved to another 29 °C incubator for 1 or 3 hours to simulate the disturbance experienced by the heat shock groups.

After the heat shock the egg containers were returned to 29 °C for a one hour ‘cool-down’ period prior to euthanasia. This ‘heat-up, cool-down’ process follows similar protocols developed for flies, frogs, lizards, and freshwater turtles (see Krebs et al., 1998; Sorensen et al., 2009; Ulmasov et al., 1992; and Ramaglia and Buck, 2004). The procedural controls also followed a mock ‘heat-up, cool-down’ regime to simulate the same movement and disturbance conditions as embryos in the heat shock treatments.

2.3.5 Heat shock treatments and euthanasia of embryos and hatchlings

Hatching success after a heat shock treatment (or procedural control) was also investigated for both Day 25 and Day 45 embryos. Eggs randomly selected
from each container for heat shocks or procedural controls on day 25 of incubation \((n = 96)\) were removed from 29 °C incubators and subjected to one of treatments T1, T2, T3, or T4. Half of these embryos \((n = 48)\) were euthanised after the heat shock by physical removal from the egg and detaching the chorio-allantoic membrane to induce respiratory and circulatory death (Deeming, 2005). Once removed from the eggs, embryos were placed onto a glass petri dish and the head was removed with a sterile scalpel blade and stored in RNALater (Life Technologies, Mulgrave, VIC, Australia) at –20 °C. The abdomen was stored at 4 °C in 100% ethanol for DNA extraction. The remaining 48 embryos were returned to the 29 °C incubator until hatching.

The same procedures were followed for eggs subjected to heat shocks or procedural controls on day 45 of incubation \((n = 96)\), except that euthanasia was achieved by physical removal from the egg and lethal injection by overdose of MS-222 into the coelom, following the protocol of Conroy et al. (2009). This anaesthetic agent was used in preference to barbiturates such as sodium pentobarbital because the integrity of genetic material is maintained (Barreto et al., 2007; Palmisano et al., 2000). Once euthanasia was confirmed by an absence of papillary response, the cervical vertebrae were severed with surgical grade bone shears. The head was removed with sterile scissors and the skullcap was peeled back to expose the brain. The brain was removed with a sterile micro-spatula and placed into RNALater to be stored at –80 °C. To access the heart, cuts were made down the coronal plane on either side of the plastron to peel away the pericardium. The heart was removed with the sterile surgical scissors and forceps and placed into RNALater to be stored at –80 °C.

The hatch date of embryos that continued incubation after the heat shock treatments or procedural controls was determined as the day that the shell first ruptured. Once hatchlings were fully emerged they were weighed and euthanised by intraperitoneal injection of a lethal dose of pentobarbitone sodium following the methodology described in Woolgar et al. (2013).
2.3.6 Total RNA extraction and quantification of Day 45 embryos

Gene expression analysis was conducted for Day 45 embryos only, as preliminary results indicated that sufficient quantities of RNA could not be extracted from Day 25 embryos. Total RNA was extracted from whole brain and heart in late embryos (FavorPrep™ Total Tissue Mini RNA Kit, Fisher Biotec, Subiaco, WA, Australia). Purity and quantity of RNA were measured with a Qubit® 2.0 Fluorometer (Invitrogen, Eugene, OR, USA). RNA quality was evaluated by gel electrophoresis on a 1.5% Agarose gel and 1× TAE Buffer. Only samples showing strong bands and quantity greater than 22.3 ng/μL were used for cDNA synthesis. A total of 24 heart samples and 29 brain samples were analysed for changes in gene expression.

2.3.7 RT-PCR primer design for 18s, Hsp60, Hsp70, Hsp90

Previously described primers for the housekeeping gene 18s (Hillis and Dixon, 1991) and heat shock protein gene Hsp60 (Kohno et al., 2010) were used to generate sequences for C. caretta. For Hsp70 and Hsp90 relevant GenBank sequences were aligned and primer pairs designed using Oligo v6.8 (Rychlik and Rhoads, 1989) [Accession numbers for sequences are listed in Table 2.1]. Total RNA was normalised to 200 ng/μl and converted to cDNA with a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer’s instructions. cDNA products for all genes were amplified using the following protocol: 10× Platinum® Supermix buffer with 5 mM dNTPs and 37.5 mM MgCl₂ plus 0.4 μM of each forward and reverse primer, 1.1 U/μL Platinum® Taq DNA Polymerase and 1 μl cDNA template in a final volume of 25 μL Q.S. with nuclease-free water. Amplification was performed using an Eppendorf MasterCycler epgradient S (Eppendorf, Hamburg, Germany) with the cycling conditions: 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 60 s, and a final extension of 72 °C for 10 min. Products were sequenced by the Australian Genome Research Facility (AGRF) on an AB 3730xl using big-dye terminator chemistry (Applied Biosystems, Foster City, CA, USA).
Table 2.1. RT-PCR sequences modified and designed for Caretta caretta-specific primer pairs for real-time PCR assays. Efficiencies and coefficients of determination are given for each primer pair.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Sequences for RT-PCR</th>
<th>Primers for qPCR</th>
<th>Annealing Temperature (°C)</th>
<th>Efficiency</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>KJ683738</td>
<td>F: GCTAATACATGCCGACGAG</td>
<td>R: GGCCCGAGGTTATCTAGAG</td>
<td>60.8</td>
<td>60.4</td>
<td>110.77%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td>KJ683735</td>
<td>F: TACTGATGCACGTGAACGCT</td>
<td>R: TGGCGTTAAGACATCTAGTG</td>
<td>59.4</td>
<td>60.5</td>
<td>103.15%</td>
</tr>
<tr>
<td>Hsp70</td>
<td>KJ683736</td>
<td>F: TCTCCGTACAGCTTGTAAGC</td>
<td>R: CCACCGAACAGATCAAGC</td>
<td>60.4</td>
<td>60.0</td>
<td>108.78%</td>
</tr>
<tr>
<td>Hsp90</td>
<td>KJ683737</td>
<td>F: GGATACTGGCATAAGGGATG</td>
<td>R: CAACACCCAAAACATGACCAATC</td>
<td>59.3</td>
<td>60.3</td>
<td>106.28%</td>
</tr>
</tbody>
</table>

2.3.8 Real-time PCR primer design and amplification of 18s, Hsp 60, Hsp70, Hsp90

A SYBR/intercalating dye was chosen to measure mRNA gene expression. Primers were designed to amplify an approximately 150bp region of the genes 18s, Hsp60, Hsp70 and Hsp90 using Oligo v6.8 (Rychlik and Rhoads, 1989) (Table 2.1). Assays were run on a StepOnePlus™ Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with iTaq Universal SYBR® Green Supermix (Bio-Rad Technologies, Gladesville, NSW, Australia) in 10 μL reactions with 1 μL cDNA template, and 0.2 μM of each forward and reverse primer. Samples were loaded in triplicate. Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s. A 3-step melt curve was generated to monitor for primer degradation in a single cycle: 95 °C for 10 s and 60 °C for 60 s, +0.3 °C to 95 °C for 15 s.
2.3.9 Data analysis

Gene expression levels were quantified with Cycle Threshold (Ct) values, which are the raw data from the real-time PCR, and refer to the number of PCR cycles required for the fluorescence signal to cross a threshold line. Ct values are inversely proportional to the amount of target nucleic acid, so the greater the Ct value, the lower the amount of target nucleic acid in the sample. All analyses were performed on mean Ct values, which were calculated from the three sample replicates used in the real-time PCR. Analysis of Variance (ANOVA) was used to test for differences in mean Ct values among treatments for each gene and tissue type combination. For these analyses, data from the two procedural control groups were pooled for each gene, and differences in expression in the four treatment groups were tested against the pooled values. Differences between pairs of treatments were assessed using unpaired t-tests. Mean Ct values were log-transformed with $2^{-\text{Ct}}$ Livak and Schmittgen (2001) prior to analysis.

Fold-change in expression levels of the target genes ($Hsp60$, $Hsp70$, and $Hsp90$) were presented using the $2^{-\Delta\Delta\text{Ct}}$ method from Livak and Schmittgen (2001), which is a standard for measuring up- or down-regulation of gene expression. The following equations explain the stepwise calculations for converting mean Ct values into fold-changes of expression:

\[
\Delta\text{Ct}_1 = \text{Ct (Target gene - treated)} - \text{Ct (Ref - treated)} \quad (1)
\]

\[
\Delta\text{Ct}_2 = \text{Ct (Target gene - control)} - \text{Ct (Ref - control)} \quad (2)
\]

\[
\Delta\Delta\text{Ct} = \Delta\text{Ct}_1 \text{ (treated)} - \Delta\text{Ct}_2 \text{ (control)} \quad (3)
\]

Normalized target gene expression level = $2^{\Delta\Delta\text{Ct}}$

All fold-change data are presented as treatment mean ± S.E. We used Chi-square analysis to test for differences in hatching success across treatments.
2.4 Results

Total amount of RNA extracted from brain tissue ranged from 202.5 to 673.2 ng, and heart tissue ranged from 217.8 to 990.0 ng, averaging 6.26 ng/mg and 5.06 ng/mg, respectively. All real-time PCR measurements for Hsp60, Hsp70, and Hsp90 were compared to the established reference gene 18s (Hillis and Dixon, 1991), which did not change mRNA expression levels across heat shock treatments in heart ($F_{4,19} = 1.30$, $P = 0.307$; Fig 2.2) or brain tissue ($F_{4,24} = 1.37$, $P = 0.273$; Fig 2.3).

![Graphs](image)

**Figure 2.2.** Comparison of 18s, Hsp60, Hsp70, Hsp90 mean Ct values (± S.E.) in whole heart tissue of Day 45 embryos for each treatment ($n = 3-5$). Statistically significant differences ($P < 0.05$) among treatment groups are indicated with dissimilar letters.
Figure 2.3. Comparison of 18s, Hsp60, Hsp70, Hsp90 mean Ct values (± S.E.) in whole brain tissue of Day 45 embryos for each treatment (n = 3-6). Statistically significant differences (P < 0.05) among treatment groups are indicated with dissimilar letters.

2.4.1 Hsp60 mRNA expression levels in heart and brain tissue

Levels of Hsp60 mRNA varied significantly among treatments in whole brain tissue ($F_{4,24} = 5.68, P = 0.002$), but not in heart tissue ($F_{4,19} = 0.74, P = 0.578$). For the whole brain tissue, pairwise $t$-tests revealed significant differences between treatment 4 (36 °C for 3 h, the highest heat shock temperature for the longest time) and all other treatments, including the control treatment. No other significant pairwise differences were detected in the whole brain tissue (Fig 2.3).
Relative to the control temperature, changes in \textit{Hsp60} mRNA expression were modest, ranging from 1.1- to 3.5-fold across treatments in both tissue types (Figs 2.4A and 2.4B).

\textbf{Figure 2.4.} Changes in levels of \textit{Hsp60}, \textit{Hsp70}, \textit{Hsp90} mRNA in whole heart tissue (A) and whole brain tissue (B) of Day 45 embryos after heat shock Treatment 1 (34 °C, 1 h), 2 (36 °C, 1 h), 3 (34 °C, 3 h), and 4 (36 °C, 3 h) as outlined in Figure 2.1 (heart \( n = 24 \); brain \( n = 29 \)). Values are presented as mean fold-change (± S.E.) relative to procedural control embryos.

\textbf{2.4.2 Hsp70 mRNA expression levels in heart and brain tissue}

There were significant differences in the relative level of \textit{Hsp70} mRNA among treatments in both heart (\( F_{4,19} = 2.92, \ P = 0.048 \)) and brain tissue (\( F_{4,24} = 3.99, \ P = 0.013 \)). For heart tissue, pairwise comparisons revealed significant differences in mean Ct values between the control and treatment 4 (36 °C for 3 h) and between treatment 1 (34 °C for 1 h, the lowest heat shock temperature
for the shortest time) and treatment 4 (Fig 2.2). For brain tissue, pairwise comparisons revealed significant differences in mean Ct values between treatment 4 and all other treatments, including the control (Fig 2.3). No other significant pairwise differences in mean Ct values were detected in either tissue.

Relative to the control, mRNA expression of Hsp70 increased from 2.7- to 38.8-fold across treatments in heart tissue (Fig 2.4A) and from 4.1 to 15.7-fold in the whole brain tissue (Fig 2.4B). For both tissues, changes in Hsp70 expression were greatest in treatments 3 (34 °C, 3 h) and 4 (36 °C, 3 h).

**2.4.3 Hsp90 mRNA expression levels in heart and brain tissue**

As found for Hsp70, there were significant differences in levels of Hsp90 mRNA among treatments in both heart ($F_{4,19} = 16.89, P < 0.001$) and brain tissue ($F_{4,24} = 24.68, P < 0.001$). For the heart tissue, pairwise comparisons revealed significant differences between the control and all heat shock treatments, as well as between treatments 1 and 4 and between treatments 3 and 4 (Fig 2.2). For brain tissue, there were significant differences between the control and all heat shock treatments, between treatments 1 and 3, between treatments 1 and 4, between treatments 2 and 4 and between treatments 3 and 4 (Fig 2.3).

Relative to the control, mRNA expression of Hsp90 increased 14.8- to 98.3-fold across treatments, with the greatest increase occurring in treatment 4 (36 °C, 3 h) (Fig 2.4A). For whole brain tissue differences in expression ranged from 3.3 to 14.7-fold across treatments. Again, the greatest increase in expression was in treatment 4 (Fig 2.4B).

**2.4.4 Hatching success of heat shocked embryos relative to controls**

Embryos from all treatments hatched after 53–57 days of incubation. The average time to hatching of embryos that experienced a heat shock on Day 25 was 56 days ($n = 21$), while the average time to hatching of embryos that experienced a heat shock on Day 45 was 55 days ($n = 33$). Hatching success of ‘Day 25 shock’ embryos averaged 65.6% across all treatments (procedural controls and heat shock treatments), while the hatching success of ‘Day 45
shock' embryos was 72.9% when averaged across treatments (Table 2.2). There was a marginally significant difference in hatching success among treatments in the Day 25 embryos exposed to a one-hour heat shock. However, there were no significant differences among treatments in the Day 25 embryos exposed to a three-hour heat shock or the Day 45 embryos (Table 2.2).

Table 2.2. Analysis of hatching success (% hatched) of heat-shocked embryos and procedural controls (treatment \( n = 16 \)).

<table>
<thead>
<tr>
<th>Day/Time</th>
<th>29 °C (procedural control)</th>
<th>34 °C</th>
<th>36 °C</th>
<th>( \chi^2 )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 25/1 h</td>
<td>56.3</td>
<td>93.8</td>
<td>56.3</td>
<td>7.0</td>
<td>0.030*</td>
</tr>
<tr>
<td>Day 25/3 h</td>
<td>75.0</td>
<td>56.3</td>
<td>56.3</td>
<td>1.6</td>
<td>0.449</td>
</tr>
<tr>
<td>Day 45/1 h</td>
<td>62.5</td>
<td>68.8</td>
<td>62.5</td>
<td>0.2</td>
<td>0.913</td>
</tr>
<tr>
<td>Day 45/3 h</td>
<td>75.0</td>
<td>93.8</td>
<td>75.0</td>
<td>2.5</td>
<td>0.292</td>
</tr>
</tbody>
</table>

* Non-significant after Bonferroni corrections.

2.5 Discussion
This study provides the first evidence that *C. caretta* embryos subjected to heat shocks upregulate their transcription of genes that encode for heat-shock proteins known to ameliorate some of the negative effects of temperature on metabolic processes. As all treatment groups successfully hatched, the heat shock treatments were non-lethal and effectively induced a thermotolerance response in the embryos. Expression differed in magnitude between heart and brain tissues, across treatments and among genes. The most severe treatment (T4; 36 °C shock for 3 h) provided the most effective set of conditions for inducing an increase in gene expression of *Hsp70* and *Hsp90*. In contrast, fold-changes were much more subdued in *Hsp60* in both tissue types, suggesting that this gene is not a good indicator of a physiological response to acute heat stress. Although we did not quantify heat-shock protein production in our study, previous studies have shown that protein expression and gene expression levels for *Hsp60*, *Hsp70*, and *Hsp90* are correlated (de Sousa Abreu et al., 2009; Ghazalpour et al., 2011), albeit with a caveat that mRNA abundances are
not direct reflections of protein abundances as most protein expression is regulated during translation or degradation (de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012). We therefore conclude that Hsp70 and Hsp90 mRNA are effective biomarkers for measuring a physiological response to heat stress in C. caretta.

2.5.1 Expression of Hsp60 in heart and brain

I found no significant changes in relative expression of Hsp60 in embryonic heart tissue in response to temperature treatments. This result implies that Hsp60 expression at the genetic level is stable during development. Hsp60 and other larger kDa proteins in the heat shock family are highly conserved across all taxa (Iwama et al., 1998), but their expression profiles can vary by tissue type and treatment design. Marber et al. (1995) demonstrated that expression of Hsp60 does not significantly alter in mammal hearts under ischemic conditions where blood flow was restricted. Later work on heart tissue of adult fresh water turtles (Chang et al., 2000; Kesaraju et al., 2009) and canines (Chang et al., 2000) in low oxygen environments also found no difference in the expression of Hsp60 between stress and basal levels. In contrast, Yan et al. (2009) found Hsp60 protein expression in the hearts of one-day old chicks (Gallus gallus) gradually increased after a 5-hour heat shock of 37 °C.

Unlike the heart tissue, I found modest yet significant changes in relative Hsp60 expression across treatments in whole brain tissue. This implies that Hsp60 expression in brain tissue has a higher temperature-sensitivity than heart tissue. Considering changes in relative expression in both tissue types, our experimental conditions produced a low to modest response to heat stress, but were significant only in brain tissue. Therefore, we conclude that Hsp60 is not well suited to act as a biomarker for thermotolerance responses to acute heat stress, but may be an effective biomarker with chronic heat stress conditions.
2.5.2 Expression of Hsp70 in heart and brain

I found that the expression of Hsp70 in embryonic C. caretta hearts was temperature-dependent and greatest at the highest temperature treatment in both tissue types. Li et al. (2012) also observed that relative expression of Hsp70 significantly increased in heart tissue of adult Chinese soft-shelled turtles (Pelodiscus sinensis) subjected to a heat shock at 40 °C for 4 hours. Not only is Hsp70 responsive to heat stress, but there is also evidence of cold sensitivity. Stecyk et al. (2012) show an increase of expression in cardiac tissue of adult freshwater turtles (Trachemys scripta) acclimated to cold (5 °C) anoxic environments. However, there were no significant changes in relative expression in telencephalon with cold acclimation (Stecyk et al., 2012). This could indicate that Hsp70 is an important protein for maintaining cardiac function under stressful conditions.

Although I found that heat shock treatments induced different levels of upregulation of Hsp70 in different tissues, increased expression relative to controls was a consistent response across all treatments and tissue types. Our observation is consistent with that of Feder and Hofmann (1999) that changes in Hsp70 expression can be used as a biological indicator of a response to thermal stress in embryonic sea turtles.

2.5.3 Expression of Hsp90 in heart and brain

Hsp90 is a chaperone protein comprising of 1–2 % of all soluble proteins in the cytoplasm (Benjamin and McMillan, 1998). Its expression is very sensitive in reptiles and can vary in both a temperature- and time-dependent manner in response to environmental stressors (Kohno et al., 2010; Ramaglia and Buck, 2004; Stecyk et al., 2012). I found significant increases in expression of Hsp90 in both heart and brain tissue, suggesting that low basal levels of the gene are active in these tissues and readily amplified in response to acute heat stress. In both tissues, expression of Hsp90 was temperature-dependent, but in brain tissue, activity was also dependent on the duration of the heat shock. Hsp90 protein is abundant in turtle brain tissue (Ramaglia and Buck, 2004), as it is necessary for protection against neuronal death (Mailhos et al., 1994; Pratt, 1998; Wyatt et al., 1996). These findings suggest that regardless of its
abundance in various tissues, \(Hsp90\) is extremely sensitive to environmental stressors during embryonic development. Therefore, it readily changes expression in response to heat stress experienced in the nest and is a relevant biomarker for determining a thermotolerance response for developing sea turtles.

### 2.5.4 Post-heat shock hatching success

Using a non-linear relationship between temperature and embryonic development rate fitted to data for Western Australian \(C. caretta\) (Woolgar et al., 2013), I calculated that Day 25 and Day 45 embryos had completed approximately 53 ± 2 % and 86 ± 2 % of their development to hatching at the time of their respective heat shocks. Hence, we exposed embryos to stressful temperatures near the sub-lethal limit of 35 °C, and within a time frame where they were potentially most vulnerable during later stages of development (Davenport, 1997).

Although there was high variation, there were no significant differences detected between the hatching success of heat shocked and control treatments for all embryos, confirming our incubation design effectively produced a stress response without causing mortality. A marginally significant effect was found in Day 25 embryos exposed to a one-hour heat shock, but the effect was no longer significant after controlling for multiple comparisons with Bonferroni corrections. It is also noteworthy that the hatching success in Day 25 embryos exposed to a one-hour heat shock did not vary between the control and the most extreme heat shock temperature and there was no significant effect in Day 25 embryos exposed to a three-hour heat shock. These results support our expectation that exposure to 34 °C is sub-lethal (Davenport, 1997). Similarly, we have evidence that brief exposure to 36 °C is also sub-lethal, though we did note that hatchlings in this treatment emerged lethargic and hunched in posture. As the natural survival rate for sea turtle embryos is around 80% (Bolten et al., 2011; Miller, 1985; Ozdemir et al., 2008), a hatching success of 75% for the most extreme temperature and duration of the heat shock indicates that this treatment produced a thermotolerance response in late-stage embryos without causing mortality.
Post-hoc analysis of the sex ratios of 119 randomly selected hatchlings from this study resulted in a 1:1 ratio of males to females (Woolgar et al., 2013). This was expected as embryos were incubated at their pivotal temperature of 29.0 °C until (1) point of sacrifice or (2) the heat shock was applied. The sex determination period for this population of *C. caretta* is between 33-64% of development to hatching stage (Woolgar et al., 2013). As our heat shocks were given either during (Day 25, 53 % developed) or after (Day 45, 86% developed) the point of gonadal differentiation, it is possible that the sex of the hatchling could have influenced gene expression. However, as the individuals for which we had gene expression data were not sexed, we were unable to test this.

2.5.5 Conclusions

Given the challenges that ecosystems face as a result of rapid environmental change, methods to assess the effects of these processes at the individual to ecosystem levels are urgently required. At the individual level, gene expression analyses are powerful tools for assessing the response of an organism to changing environments. By simulating acute heat stress in *C. caretta* embryos, we successfully elicited a heat stress response above a control group. Relative expression of *Hsp70* and *Hsp90* significantly increased in both heart and brain tissue in reaction to heat stress, and we conclude that these genes are appropriate biomarkers for measuring a thermotolerance response in *C. caretta*. In contrast, *Hsp60* mRNA was not an effective biomarker in our experimental design. Our results serve as a platform for further studies of clutch- and population-level variation in this species, and potentially other species of sea turtle.

The management implications of my research relate not only to climate change and increasing sand temperatures on the natal beaches of sea turtles, but also to anthropogenic alteration of beach temperatures via removal of vegetation or changes in sand composition (Fuentes et al., 2011; Hawkes et al., 2007). Higher incubation temperatures may have profound impacts on hatching success (Birchard, 2005), but this study demonstrates that embryonic *C. caretta* have mechanisms that offer some resilience to thermal stress, which may allow
them to survive in a warmer nest environment. The extent of this resilience requires further research to investigate how proteins are upregulated under higher or longer periods of heat stress, and whether hatching success is compromised under more extreme conditions. The capacity for reptile embryos to acclimate to warmer nest environments through changes in their phenotype is an important area for further study given the current pace of environmental change.
2.6 References


Chapter 3: Reconstructed paternal genotypes reveal variable rates of multiple paternity at three rookeries of loggerhead sea turtles (Caretta caretta) in Western Australia

NB: This chapter has been published in Australian Journal of Zoology and some of the formatting requirements for that journal have been maintained. Co-authors have been acknowledged in the Declarations.
3.1 Abstract
Female sea turtles are promiscuous, with clutches of eggs often sired by multiple males and rates of multiple paternity varying greatly within and across species. I investigated levels of multiple paternity in loggerhead sea turtles (*Caretta caretta*) from three rookeries in Western Australia by analysing polymorphic species-specific genetic markers. It was predicted that level of multiple paternity would be related to female population size and hence the large rookery at Dirk Hartog Island would have higher rates of multiple paternity than two smaller mainland rookeries at Gnaraloo Bay and Bungelup Beach. Contrary to my prediction, I found highly variable rates of multiple paternity among the rookeries that I sampled, which was unrelated to female population size (25 % at Bungelup Beach, 86 % at Gnaraloo Bay, and 36 % at Dirk Hartog Island). Approximately 45 different males sired 25 clutches and the average number of sires per clutch ranged from 1.2 to 2.1, depending on the rookery sampled. The variance in rates of multiple paternity among rookeries suggests that operational sex ratios are variable in Western Australia. Periodic monitoring would show if the observed patterns of multiple paternity for these three rookeries are stable over time, and these data provide a baseline for detecting shifts in operational sex ratios.

3.2 Introduction
Unlike many bird and mammal species, parental care beyond nesting is absent in most reptiles (Shine 2005; Uller and Olsson 2008). Males do not provide any resources to females other than sperm, yet multiple paternity in clutches has been recorded in most reptile species to date (Uller and Olsson 2008). Multiple paternity has been detected in all seven extant species of sea turtle, with one or two fathers being the most common number of sires of a single clutch (reviewed by Bowen and Karl 2007 and Lee 2008). In sea turtles, multiple paternity can arise in two ways: either a female can mate with more than one male during the same reproductive cycle, or alternatively, a female may utilise sperm stored from a previous breeding season (Pearse and Avise 2001; Lara-De La Cruz et al. 2010; Phillips et al. 2014a).
Many explanations for multiple paternity have been proposed, including increased fertilisation success, improved offspring fitness, and male harassment of receptive females (Jensen et al. 2013). Ireland et al. (2003) and Lee and Hays (2004) suggested that the phenomenon was a product of male density and female avoidance of aggressive mating behaviour, causing females to mate with more than one male (convenience polyandry). A study on solitary and mass-nesting (arribada) olive ridley turtles (Lepidochelys olivacea) by Jensen et al. (2006) attributed the higher rate of multiple paternity in the arribada females to their high density of nesting. As males rarely come ashore and are difficult to catch at sea, genetic analyses of nesting females and their offspring can both identify the number of fathers per clutch and provide data on the number of breeding males and females from which operational sex ratios (OSRs) can then be calculated (Wright et al. 2012a, 2012b; Hawkes et al. 2014). The OSR of a given population should be proportional to the number of males at the breeding area prior to the nesting season (Hays et al. 2010; Stewart and Dutton 2011), and therefore reflect the underlying genetic variation of the population.

Relative to other parts of the world, little is known about the population dynamics of loggerhead turtles (Caretta caretta) nesting in the eastern Indian Ocean. In Australia, there are two genetically distinct populations of C. caretta, one in Western Australian (WA) and the other in Queensland (Baldwin et al. 2003). All rookeries in WA comprise a single genetic stock (Pacioni et al. 2012), spanning approximately 520 km of coastline from Dirk Hartog Island (25.49827 °S, 112.98719 °E) at the southern limit to the Muiron Islands northeast of Exmouth (21.39156 °S, 114.21205 °E) at the northern limit of the range (Baldwin et al. 2003). Although the rookeries within this area constitute the third-largest population of C. caretta in the world (Baldwin et al. 2003; Reinhold and Whiting 2014), relatively little is known about the population demographics. A description of mating systems, quantification of the incidence of multiple paternity, and quantification of genetic variation is a first step toward understanding the implications of climate change and changing sex ratios of this globally important population.
Genetic analyses offer a means to indirectly sample the male component of a population of breeding turtles (Lee 2008; Phillips et al. 2014b; Stewart and Dutton 2011) and there are several methods for estimating multiple paternity using genetic data (Table 3.1). For sea turtles, such studies show that rates of multiple paternity are highly variable (Table 3.1), though it is unclear whether the reported variability among species and populations is due to the use of different types of genetic markers, differences in multiple paternity estimation methods, or if indeed it reflects natural variability among populations. To date, only one study (Jensen et al. 2006) has concurrently examined the frequency of multiple paternity in two different rookeries of the same species.
Table 3.1. Variation in rates of multiple paternity in sea turtles within species and across studies.

<table>
<thead>
<tr>
<th>Location</th>
<th>No. clutches analysed</th>
<th>Mean no. offspring genotyped per clutch</th>
<th>No. loci analysed</th>
<th>Min. no. males</th>
<th>Freq. MP</th>
<th>Methods</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green (C. mydas)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascension Island</td>
<td>18</td>
<td>38.9</td>
<td>2-5</td>
<td>2</td>
<td>61%</td>
<td>DADSHARE, GERUD</td>
<td>Lee &amp; Hays, 2004</td>
</tr>
<tr>
<td>Ascension Island</td>
<td>3</td>
<td>15.3</td>
<td>2</td>
<td>2</td>
<td>100%</td>
<td>REAP</td>
<td>Ireland et al., 2003</td>
</tr>
<tr>
<td>Southern GBR</td>
<td>22</td>
<td>41.3</td>
<td>5</td>
<td>1</td>
<td>9%</td>
<td>GENEPOP</td>
<td>FitzSimmons, 1998.3.1</td>
</tr>
<tr>
<td>Tortuguero, Costa Rica</td>
<td>8</td>
<td>—</td>
<td>2</td>
<td>2</td>
<td>63%</td>
<td>Irwin</td>
<td>Peare &amp; Parker, 1996</td>
</tr>
<tr>
<td>Algadi, Cyprus</td>
<td>20</td>
<td>21.9</td>
<td>14</td>
<td>1.4</td>
<td>36%</td>
<td>COLONY 2.0</td>
<td>Wright et al., 2012b</td>
</tr>
<tr>
<td>Algadi, Cyprus</td>
<td>94</td>
<td>21.7</td>
<td>13</td>
<td>1.1</td>
<td>23%</td>
<td>COLONY 2.0</td>
<td>Wright et al., 2012a</td>
</tr>
<tr>
<td>Location</td>
<td>Sample</td>
<td>Nests</td>
<td>Hatching Mortality</td>
<td>Mortality</td>
<td>Method</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>--------</td>
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<td>--------------------</td>
<td>-----------</td>
<td>-------------</td>
<td>----------------------------</td>
<td></td>
</tr>
<tr>
<td>Sri Lanka</td>
<td></td>
<td>24</td>
<td>6</td>
<td>1.7</td>
<td>63%</td>
<td>GERUD 2.0</td>
<td>Ekanayake et al., 2013</td>
</tr>
</tbody>
</table>

**Loggerhead (C. caretta)**

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample</th>
<th>Nests</th>
<th>Hatching Mortality</th>
<th>Mortality</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zakynthos, Greece</td>
<td>15</td>
<td>40.7</td>
<td>4</td>
<td>3.2</td>
<td>93%</td>
<td>GERUD 1.0</td>
</tr>
<tr>
<td>Melbourne Beach, Florida</td>
<td>70</td>
<td>10</td>
<td>4</td>
<td>1.4</td>
<td>31%</td>
<td>PARENTAGE</td>
</tr>
<tr>
<td>Mon Repos, Queensland</td>
<td>24</td>
<td>21</td>
<td>0</td>
<td>–</td>
<td>33%</td>
<td>Allozymes</td>
</tr>
<tr>
<td>Melbourne Beach, Florida</td>
<td>3</td>
<td>20.7</td>
<td>2</td>
<td>–</td>
<td>33%</td>
<td>–</td>
</tr>
<tr>
<td>Nagoya, Japan</td>
<td>7</td>
<td>29</td>
<td>2-Aug</td>
<td>–</td>
<td>43%</td>
<td>–</td>
</tr>
</tbody>
</table>

**Olive ridley (L. olivacea)**

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample</th>
<th>Nests</th>
<th>Hatching Mortality</th>
<th>Mortality</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ostional, Costa Rica</td>
<td>13</td>
<td>22</td>
<td>2</td>
<td>2.8</td>
<td>92%</td>
<td>GERUD 1.0</td>
</tr>
<tr>
<td>Playa Hermosa, Costa Rica</td>
<td>13</td>
<td>22.6</td>
<td>2</td>
<td>1.4</td>
<td>30%</td>
<td>GERUD 1.0</td>
</tr>
<tr>
<td>Galibi, Suriname</td>
<td>10</td>
<td>70.3</td>
<td>2</td>
<td>1.2</td>
<td>20%</td>
<td>Initial</td>
</tr>
</tbody>
</table>

\[\text{inference}\]
<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Eggs</th>
<th>Hatching</th>
<th>Survival</th>
<th>Tool</th>
<th>Study Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kemp's ridley</strong> (L. kempi)**</td>
<td>Tamaulipas, Mexico</td>
<td>26</td>
<td>7.8</td>
<td>3</td>
<td>–</td>
<td>58%</td>
</tr>
<tr>
<td><strong>Hawksbill</strong> (E. imbricata)**</td>
<td>Gulisaan, Sabah, Malaysia</td>
<td>10</td>
<td>27</td>
<td>3</td>
<td>1.3</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>Cousine Island, Seychelles</td>
<td>43</td>
<td>18.8</td>
<td>33</td>
<td>–</td>
<td>9.3%</td>
</tr>
<tr>
<td></td>
<td>Seychelles (various islands)</td>
<td>249</td>
<td>22.6</td>
<td>32</td>
<td>–</td>
<td>9.2%</td>
</tr>
<tr>
<td><strong>Leatherback</strong> (D. coriacea)**</td>
<td>Las Baulas, Costa Rica</td>
<td>4</td>
<td>–</td>
<td>2</td>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Sandy Point, Virgin Islands</td>
<td>38</td>
<td>26.8</td>
<td>7</td>
<td>–</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td>Sandy Point, Virgin Islands</td>
<td>17</td>
<td>10.5</td>
<td>6</td>
<td>–</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Playa Grande, Costa Rica</td>
<td>20</td>
<td>19.5</td>
<td>3</td>
<td>–</td>
<td>10%</td>
</tr>
<tr>
<td><strong>Flatback</strong> (N. depressus)**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mon Repos & Peak Island, Qld 16 26.7 4 – 69% Initial Theissinger et al., 2009

inference,

Chi-sq,

PARENTAGE

1.0, GERUD

2.0, MER 3.0

Arribada nesting beach; a Solitary nesting beach; c Captive population, paternal genotype known

a
Here, I investigated patterns of multiple paternity in clutches sampled from three locations spread across the geographic range of rookeries of *C. caretta* in WA. The southern-most rookery was on Dirk Hartog Island, one of the world’s largest rookeries (Baldwin et al. 2003; Reinhold and Whiting 2014), whilst I also sampled clutches from near the northern-most edge (Bungelup Beach) and from a smaller mainland rookery approximately mid-way in the breeding range (Gnaraloo Bay). I aimed to describe: 1) the presence of multiple paternity, and 2) spatial variation in multiple paternity rates among rookeries across the range of the nesting population. Because these rookeries differed in size, I predicted that the frequency of multiple paternity should be higher in clutches from the larger nesting rookery at DHI compared to the smaller, mainland rookeries (GB and BB) based on the density-dependence convenience polyandry model. To exclude the possibility that any variation in multiple paternity I detected was an artefact of methodology, I analysed paternity using identical statistical methods and the same genetic markers for samples from all rookeries. The results are discussed in the context of estimating population size and the implications of climate change on the demography of the WA population of *C. caretta*.

### 3.3 Methods

#### 3.3.1 Egg collection and tissue sampling

Eggs of *C. caretta* were collected from three rookeries in WA during peak nesting periods between 2011 and 2013. Collection sites and dates were Turtle Bay on Dirk Hartog Island (25.49827 °S, 112.98719 °E) in January 2013, Gnaraloo Bay on the WA mainland (23.82618 °S, 113.52629 °E) in January 2011 (Woolgar et al. 2013), and Bungelup Beach in the Cape Range National Park on the Exmouth Peninsula (22.282331 °S, 113.831570 °E) in December 2013 (Fig 3.1). The Dirk Hartog Island rookery hosts the largest nesting numbers, with approximately 2000 nesting females per season (Trocini 2013; Reinhold and Whiting 2014), while an estimated 700-1200 females nest per season at Bungelup Beach (Trocini 2013). In contrast, nesting at the Gnaraloo Bay rookery is comparatively infrequent with approximately 100 females per season (Hattingh et al. 2011).
Figure 3.1. Locations of the three collection sites: Dirk Hartog Island, Gnaraloo Bay, and Bungelup Beach. More than 2000 females nest per season on Dirk Hartog Island, most notably on Beach 1 and Beach 5. Map adapted from Trocini (2013), and Reinhold and Whiting (2014).
All offspring samples in this study were used opportunistically, as they were collected for other research projects (Woolgar et al. 2013; Tedeschi et al., unpublished data). As a consequence, sample sizes varied among clutches and among rookeries. I had access to 80 eggs per clutch \((n = 15\) clutches) from the Dirk Hartog rookery, and 20 eggs per clutch \((n = 4\) clutches) from Bungelup Beach. Eggs sampled from these rookeries were incubated in the laboratory and embryos were euthanised prior to hatching. Maternal tissue was collected from these two rookeries during oviposition by sampling from the trailing edge of the back flipper with a sterile 3 mm biopsy punch (Bydand Medical, NSW, Australia). At the Gnraloo Bay rookery, hatchlings were collected from nests for a study conducted by Woolgar et al. (2013), and I used available samples to assess paternity (GB; \(n = 10–22\) eggs per clutch from 8 clutches). No maternal tissue samples were available for the GB rookery because the collection permit for the study on this population did not cover sampling of adult females. All samples from the DHI \((n = 859)\) and BB \((n = 66)\) rookeries were stored at room temperature in 2 mL Longmire buffer until processing, whereas samples from the GB rookery \((n = 119)\) were stored at 4 °C in 1.5–2.0 mL of 100 % EtOH.

### 3.3.2 Microsatellite analysis and genotyping

Fourteen of the 15 clutches collected from DHI were genotyped, as one clutch was unfertilised. Total DNA was extracted from 1026 samples of offspring (minimum of 10 offspring per clutch) and 18 maternal samples using a standard salting out method (Sunnucks and Hales 1996), with the exception of proteinase K digestion [200 µg/ml] at 56 °C overnight. The DNA pellet was resuspended in 100 µL nuclease-free sterile water and quantified by NanoDrop® Spectrophotometer (ND1000, Thermo Fisher Scientific, Australia). All samples were normalised to 10 ng DNA per µL with nuclease-free water prior to polymerase chain reaction (PCR).

Four loci designed for *C. caretta*, Cc8E07, Cc7B07, Cc5F01, Cc7C04 (see Shamblin et al., 2007) were run in a single PCR multiplex. PCR was performed in 10 µL reactions with 1 ng DNA template, 7.8 µL Platinum Supermix (Invitrogen, Life Technologies, VIC, Australia), 0.2 µL MgCl2 [50mM], and 0.25 µL each primer [7µM]. PCR products were denatured at 95 °C for 3 mins, (40x)
30 s at 95 °C, 45 s at 53 °C, 30 s at 72 °C, and 8 min extension at 72 °C. All PCR products were analysed on an ABI 3730 Sequencer against GeneScan 500 LIZ internal size standard and DNA fragments were scored manually with GeneMarker v1.91 software (SoftGenetics, LLC®, USA).

### 3.3.3 Data analysis

Levels of genetic variation among the 18 maternal genotypes (DHI and BB rookeries) were assessed by calculating the number of alleles per locus, and allele frequencies at each locus using the GENEALEX 6.5 software package (Peakall and Smouse 2012). I also used this program to assess Hardy-Weinberg equilibrium and calculate the probability of two different females having identical multilocus genotypes. Observed and expected heterozygosity for the four loci for each rookery were estimated with CERVUS 3.0.3 (Marshall et al. 1998). The presence of null alleles was tested at each locus in only the 14 maternal genotypes from the DHI rookery using the software package MICROCHECKER (Van Oosterhout et al. 2004); the sample size from the BB was insufficient for detecting null alleles with reliability.

I assessed paternity within each clutch sample using initial inference, the GERUD 2.0 software package (Jones 2005), and the COLONY 2.0 software package (Wang 2004; Wang and Santure 2009). Neither GERUD 2.0 nor COLONY 2.0 require population allele frequencies in order to calculate the minimum number of fathers (Jones 2005; Wang and Santure 2009), so these packages were ideal for my purposes given that other adult females from the rookeries were not sampled.

To evaluate paternity with initial inference I used the maternal genotypes to identify maternal contributions to each offspring and inferred paternal alleles by excluding maternal alleles in the offspring genotypes (Jones et al. 2010). Multiple paternity was determined when three or more non-maternal alleles were found at a single locus. Since maternal genotypes were not available for the GB rookery, I inferred maternal allelic contribution based on the frequency of alleles in the offspring within each clutch. The GERUD 2.0 analyses were performed using all four loci with the parameter for the maximum number of
fathers set to four. Runs were conducted with and without maternal genotypes. When the GERUD program returned multiple solutions for progeny arrays, they were ranked by likelihood based on the segregation of paternal alleles and their deviation from Mendelian expectations (Jones 2005). The combination of fathers with the highest probability score was used to calculate the minimum number of fathers for the clutch.

The COLONY analyses were also performed using all four loci. COLONY assigns sibships and parentage based on a maximum likelihood model. Offspring are clustered by full-sib and half-sib (maternal and paternal), and parent-offspring relationships are determined with parents assigned to full-sib groups. Unknown genotypes for either parent can be inferred (Wang 2004; Wang and Santure 2009). For each rookery, all genotyped offspring were analysed in a single dataset to identify any paternal half-sibs, which would indicate males that sired offspring with more than one female. COLONY was set to the default parameters, a single medium-length run, with full-likelihood analysis, assuming polygamy for both males and females. Parallel to the GERUD analysis, COLONY runs were performed with and without maternal genotypes.

COLONY can estimate paternity with datasets containing missing or rare alleles, but GERUD cannot. Offspring that lacked maternal alleles or had missing data, were therefore excluded from the GERUD analysis. The reduced dataset for the DHI rookery included genotypes for 14 females and 791 offspring; full dataset included 813 offspring. For BB, the reduced dataset was for 4 females and 60 offspring; full dataset included 62 offspring. Finally, for the GB rookery, the reduced dataset included 84 offspring while the full dataset analysed included 92 offspring. Following the consensus approach proposed by Theissinger et al. (2009) and Stewart and Dutton (2011), multiple paternity was identified in each clutch if two of the three methods used had detected more than one father.
3.4 Results

All four loci were polymorphic, with the number of alleles per locus ranging from 6 to 22 with observed heterozygosity ranging from 0.70 to 1.00 (Table 3.2). The probability of females from the BB and DHI rookeries sharing a multilocus genotype ranged from $1.6 \times 10^{-2}$ to $7.5 \times 10^{-2}$. Genotypic frequencies for the DHI rookery at all loci were in agreement with Hardy-Weinberg equilibrium ($P > 0.05$), and no null alleles were detected.

Table 3.2. Descriptive statistics of the four polymorphic microsatellite markers. $n$ is the sample size, $A$ is the mean number of alleles per locus, $H_O$ is observed heterozygosity and $H_E$ is expected heterozygosity.

<table>
<thead>
<tr>
<th>Rookery</th>
<th>$n$</th>
<th>Locus</th>
<th>Allele size range (bp)</th>
<th>$A$</th>
<th>$H_O$</th>
<th>$H_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dirk Hartog (DHI)</td>
<td>791</td>
<td>Cc8E07</td>
<td>248-299</td>
<td>13</td>
<td>0.901</td>
<td>0.873</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc5F01</td>
<td>115-178</td>
<td>18</td>
<td>0.886</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7C04</td>
<td>184-233</td>
<td>14</td>
<td>0.804</td>
<td>0.862</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7B07</td>
<td>212-304</td>
<td>22</td>
<td>0.833</td>
<td>0.915</td>
</tr>
<tr>
<td>Bungelup (BB)</td>
<td>60</td>
<td>Cc8E07</td>
<td>248-291</td>
<td>9</td>
<td>0.883</td>
<td>0.827</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc5F01</td>
<td>116-169</td>
<td>11</td>
<td>0.967</td>
<td>0.891</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7C04</td>
<td>192-233</td>
<td>6</td>
<td>0.700</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7B07</td>
<td>216-304</td>
<td>13</td>
<td>1.000</td>
<td>0.909</td>
</tr>
<tr>
<td>Gnaraloo (GB)</td>
<td>84</td>
<td>Cc8E07</td>
<td>248-315</td>
<td>13</td>
<td>0.833</td>
<td>0.885</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc5F01</td>
<td>115-190</td>
<td>15</td>
<td>0.917</td>
<td>0.929</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7C04</td>
<td>188-233</td>
<td>11</td>
<td>0.905</td>
<td>0.844</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cc7B07</td>
<td>216-308</td>
<td>17</td>
<td>0.940</td>
<td>0.911</td>
</tr>
</tbody>
</table>
The estimated proportions of multiple paternity varied among rookeries (Table 3.3). Based on initial inference, the frequency of multiple paternity was 25.0 % (1/4 clutches) at BB, 35.7 % (5/14 clutches) at DHI and 85.7 % (6/7 clutches) at GB. The mean minimum number of fathers per clutch estimated using initial inference ranged from 1.2 to 1.9 (Table 3.3).
Table 3.3. Minimum number of fathers per clutch in *C. caretta* as estimated by initial inference, GERUD 2.0, and COLONY 2.0 runs with and without maternal genotype. Multiple paternity was concluded when at least two of the three methods detected a minimum of two fathers per clutch, shown in bold.

<table>
<thead>
<tr>
<th>Rookery</th>
<th>Clutch</th>
<th>No. Embryos Analysed (Clutch Size)</th>
<th>Initial Inference</th>
<th>GERUD</th>
<th>COLONY</th>
<th>Multiple Paternity?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHI</td>
<td>A</td>
<td>41 (45)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>60 (69)</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>37 (42)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>45 (46)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>52 (58)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>53 (62)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>% MP</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
<td>25% (1/4)</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mean</td>
<td>1.25 ± 0.25</td>
<td>1.25 ± 0.25</td>
<td>1.25 ± 0.25</td>
<td>1.25 ± 0.25</td>
<td>1.25 ± 0.25</td>
<td>1.25 ± 0.25</td>
</tr>
<tr>
<td>(GB)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>14 (15)</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10 (11)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>12 (12)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10 (10)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>13 (20)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>13 (15)</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>12 (14)</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>% MP</td>
<td>71.4% (5/7)</td>
<td>100% (7/7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42.9% (3/7)</td>
<td>85.7% (6/7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Percentages (% MP) and mean values ± S.E.M. of estimated rates of multiple paternity are indicated for each method across all clutches analysed. Estimates of multiple paternity and the minimum number of fathers per clutch were slightly higher when calculations were based on the GERUD and COLONY analyses. The frequency of multiple paternity ranged from 25 % (1/4) to 100 % (7/7) and the minimum number of fathers per clutch ranged from 1.1 to 2.1 (Table 3.3). Nevertheless, a similar pattern to the initial inference estimates was apparent, with both estimates for the large rookery at DHI being closer to the lower range values. The GERUD estimates of minimum number of fathers per clutch and frequency of multiple paternity were identical when calculated with or without maternal genotypes. Two additional instances of multiple paternity were detected in the COLONY analyses when runs were conducted without maternal genotype (Table 3.3).

Reconstructed paternal genotypes from GERUD and COLONY agreed six out of the 12 instances (50%) where multiple paternity was determined across the three rookeries. The analyses indicated that 16 to 25 individual males sired offspring in the clutches sampled from the DHI rookery (n = 14), 5 males sired offspring in the clutches from BB (n = 4), and 11 to 15 males sired offspring in the clutches from the GB rookery (n = 7). None of the males were identical across the three rookeries. Where maternal genotypes were known, the probability of two males from the BB and DHI rookeries sharing a multilocus genotype ranged from 5.5x10^{-2} to 9.2x10^{-3}. Where the maternal genotype was not known, the probability of two males from all three rookeries sharing a multilocus genotype ranged from 1.6x10^{-2} to 9.2x10^{-3}.

3.5 Discussion
At three *C. caretta* rookeries in Western Australia, females laid clutches that were sired by multiple males 25-86 % of the time during peak nesting periods between 2011 and 2013. This result is consistent with estimates of multiple paternity in populations of *C. caretta* from the Northern Hemisphere and eastern Australia, where rates of 25-33 % are typical (Table 3.1).
The highest rate of multiple paternity was found at Gnaraloo Bay (GB) where multiple males sired 86% of clutches (assuming paternal genotypes were correctly deduced from correctly inferred maternal genotypes). It is unclear why such a high rate of multiple paternity should occur in a low-nesting rookery such as GB, although the size of the offshore breeding area occupied by males and females may impact the nesting density, and hence male/female encounters. For example, Zbinden et al. (2007) reported that 93% of C. caretta clutches from the Laganas Bay rookery on Zakynthos Island in Greece exhibited multiple paternity. They attributed this rate to the small size of the bay that bordered the nesting beach, which confined the population and increased densities of breeding males and females. Lasala et al. (2013) also report a high rate of multiple paternity (75%) for nests on Wassaw Island, Georgia. The authors suggest this may be due to a large number of males migrating along the coastline and crossing nesting beach boundaries. The GB rookery is situated on a wide and open bay with near-continuous fringing reef (Short 2005; Hattingh et al. 2011), so it is plausible the high rate of multiple paternity found at this rookery is a result of large numbers of males migrating along the fringing reefs. However, as it is now known how much of the offshore area comprises the breeding grounds, tracking of sea turtles in the water during the breeding season would indicate the density of turtles at sea, and permit estimation of the probability of male/female encounters (Schofield et al. 2013).

In contrast to the rookery at GB, the frequency of multiple paternity was lower in clutches sampled from the BB (25%) and DHI (36%) rookeries. Although the estimate of low multiple paternity for the BB rookery may reflect the relatively small clutch and offspring sample sizes, this was not the case for DHI, where sample sizes and the number of clutches analysed were comparatively large (see Table 3.1). Uller and Olsson (2008) proposed that all else being equal, the degree of multiple paternity should be positively correlated with the probability of mate encounters. If this model applies to C. caretta in Western Australia, it would imply that male density is higher in the centre of the species WA distribution. However, additional survey and molecular work would be required to verify this possibility.
I found no evidence of a relationship between rookery size and the incidence of multiple paternity as has been reported elsewhere in sea turtles (Lee 2008). The *C. caretta* rookery at DHI is one of the largest in the world (Baldwin et al. 2003; Reinhold and Whiting 2014), but has one of the lowest rates of multiple paternity reported. Phillips et al. (2013, 2014b) found a similar pattern in a population of hawksbill turtles (*Eretmochelys imbricata*) nesting in the Seychelles Islands. They found a high number of males contributing to the clutches sampled (47 males fertilised 43 clutches), but the frequency of multiple paternity was low (9.3 %), which they attributed to a low rate of mate encounter over a widely dispersed breeding area (Phillips et al. 2013, 2014b). The lack of a relationship between rookery size and rates of multiple paternity might also reflect a declining number of males associated with feminization of primary sex ratios due to climate change (Wright et al. 2012b; Hawkes et al. 2014). However, there is no data on the trends in male abundance in this genetic stock to support this assumption.

If rookery topography is not a factor in determining the frequency of multiple paternity at GB, perhaps population demographics can explain this high estimate. FitzSimmons et al. (1997a) found that both male and female green turtles (*C. mydas*) in eastern Australia exhibit similar levels of philopatry to their native beaches. If this behaviour is also common to loggerhead turtles in Western Australia, the high rate of multiple paternity occurring at the GB rookery may reflect a greater number of males returning to breed than at the DHI and BB rookeries. Alternatively, more male offspring may be produced at the GB rookery. Tentative support for this idea comes from a study that compared empirical and modelled nest temperatures at each of the three study rookeries, where the mid-range GB rookery had cooler beach temperatures relative to the two range-edge rookeries at DHI and BB (Woolgar 2012). Hence, as *C. caretta* has temperature-dependent sex determination, with males being produced at cooler incubation temperatures, (Mrosovsky 1994; Miller 1985; Standora and Spotila 1985), the GB rookery, at the centre of the species’ range, may produce relatively more male offspring than at the other two rookeries studied (see Woolgar et al. 2013). This in turn could drive differences in the operational sex ratios (ORSs) of each nesting population. Further, male-
mediated gene flow is promoted by mating on migration routes and possibly feeding grounds (FitzSimmons et al. 1997b), which may contribute to sex ratio differences between rookeries, especially if females and males travel different routes (FitzSimmons et al. 1997b; Wright et al. 2012b). Measuring nest incubation temperatures across years to assess long-term changes in hatchling sex ratios (Laloë et al. 2014) in combination with long-term genetic monitoring of the nesting females will show if the pattern I observed for these three rookeries is temporally stable, and this data can be used as a baseline for determining if OSRs change over time.

Two common methods for estimating OSRs in sea turtle populations are to count the number of females and males encountered along a transect (Hays et al. 2010) or to estimate paternal contributions of clutches sampled from nesting beaches (Stewart and Dutton 2001; Hawkes et al. 2014). OSRs change as the nesting season progresses, as males and females arrive at breeding grounds at different times, have different periods of residence, and different remigration intervals (Limpus 1993; Godley et al. 2002; Hays et al. 2010, 2014). As the remigration interval for male *C. caretta* is shorter than for females, future scenarios of climate change may not decrease population viability even with increased feminisation of offspring (Hays et al. 2010; Phillips et al. 2014b, but see Wright et al. 2012a). As long as males return frequently to breeding grounds, fertilisation success should be stable (Hays et al. 2010; Wright et al. 2012b). Hence, healthy and genetically diverse populations should be able to absorb a reduction in males given the polyandrous nature of sea turtles, but periodic monitoring of OSRs (e.g. every 5 to 10 years) is critical for detecting ratios that could reduce population viability.

In summary, it is clear that multiple paternity is the normal mating system in most species of sea turtle, and that rates vary by species, population, and by the method of detection (Bowen and Karl 2007). Despite the lack of a maternal genotype for one of the three rookeries, all methods used in this study led us to conclude that multiple paternity rates ranged from 25 to 86 % in *C. caretta* clutches sampled from WA rookeries. Additional samples from BB and GB (including maternal tissue), as well as from the Muiron Islands at the northern
limit of the nesting range would be valuable for assessing whether my estimates of the rates of multiple paternity are representative of the WA population. Further, reconstruction of the genotypes of males that successfully mated with females, as I did in this study, allows the indirect estimation of the number of males contributing to this population and hence more realistic estimation of the adult population size. The rates of multiple paternity have detected provide a snapshot of the mating system of the WA population, and it will be important to repeat sampling in order to detect changes in OSRs over time.
3.6 References


Woolgar, L. (2012). A comparison of two techniques used to model sand temperatures and sex ratios at loggerhead turtle (Caretta caretta) rookeries in Western Australia. Master's Thesis, The University of Western Australia, Perth.


Chapter 4: Heritable variation in heat shock gene expression: a potential mechanism for adaptation to thermal stress in embryos of sea turtles

NB: This chapter has been submitted to *Proceedings B* and some of the formatting requirements for that journal have been maintained. Co-authors have been acknowledged in the Declarations.

4.1 Abstract

The capacity of species to respond adaptively to warming temperatures will be key to their survival in the Anthropocene. The embryos of egg-laying species such as sea turtles have limited behavioural means for avoiding high nest temperatures, so responses at the molecular level may be critical to coping with
predicted global temperature increases. Using the loggerhead turtle (Caretta caretta) as a model, I used quantitative PCR to characterise variation in the expression response of heat shock genes (hsp60, hsp70, and hsp90; molecular chaperones involved in cellular stress response) to an acute non-lethal heat shock. Result show significant variation in gene expression at the clutch and population levels for some, but not all hsp genes. I then used pedigree information to estimate heritabilities of the expression response of hsp genes to heat shock and demonstrate both maternal and additive genetic effects. This is the first evidence for heritability of heat shock responses in sea turtles and at the embryonic stage in any reptile. The presence of heritable variation in the expression of key thermotolerance genes is necessary for sea turtles to adapt at a molecular level to warming incubation environments.

4.2 Introduction

Survival in a warming world depends on the ability of a species to move or adapt to climate change (Skelly et al. 2007; Hoffmann & Sgrò 2011; Bellard et al. 2012). Species may adapt at a physiological level by evolving tolerance to warmer climates, or by modifying life history strategies (Bellard et al. 2012). In particular, phenotypic plasticity of physiological responses should maximise the possibility of species persisting in their current environments (Urban et al. 2014).

Physiological responses to stress begin at the molecular level with the activation of genes such as those that code for heat shock proteins, which mitigate damage to membranes, proteins, and genomic material (Feder & Hofmann 1999). The larger heat shock proteins of the Hsp60, Hsp70, and Hsp90 families increase expression in response to heat stress (Feder & Hofmann 1999; Kültz 2005) and act to maintain protein folding and degradation (Sørensen et al. 2003) and myelination of neurons to avoid apotosis (Mailhos et al. 1994). The upregulation of heat shock protein genes (hsp) in response to thermal stress is highly conserved across taxa [e.g. insects (Krebs et al. 1999; Hoffmann et al. 2003), fish (Fangue et al. 2006), frogs (Sørensen et al. 2009), fresh water turtles (Stecyk et al. 2012), embryonic sea turtles (Chapter 2)], and
is therefore a candidate genomic mechanism for adaptation to climate change (Howard et al. 2014).

The mechanisms by which sea turtle embryos respond to heat stress are of particular interest, as their lineage has persisted through many instances of global heating and cooling (Pritchard 1997; Hamann et al. 2007; Poloczanska et al. 2009). However, the strong fidelity of nesting females to natal beaches (Lohmann et al. 2013) means that embryos could be subject to unprecedentedly rapid increases in nest temperatures under anthropogenically-forced climate change (Hamann et al. 2013; Pike 2014).

Increased expression of heat shock genes may provide a mechanism for sea turtle embryos to adapt to higher incubation temperatures. For this to be possible, two key expectations need to be met. First, geographic variation in expression should exist between contemporary populations experiencing different thermal nesting environments (Pörtner 2002; Deutsch et al. 2008). Second, and more importantly, the variation in expression of hsp genes in response to thermal stress must be heritable (Feder 1999). In general there have been few studies on the adaptive potential of wild populations to heat stress, and laboratory evolution experiments have focused on the heritability of expression of hsp70 as a response in Drosophila (McColl et al. 1996; Bettencourt et al. 1999). Given that Drosophila have very short generation times (a few weeks), low levels of heritability may be sufficient for adaptation to rapid warming. In contrast, sea turtles have generation times of approximately 20-30 years (Heppell et al. 2003), which constrains the rate at which nesting behaviour could evolve to achieve optimal incubation environments for developing embryos. This leaves sea turtle embryos with few options to avoid increasing nest temperatures, and the extent to which expression of heat shock genes is heritable will affect the evolution of their critical thermal limits.

Here, I investigated phenotypic and genetic variation of hsp gene expression in the embryos of loggerhead sea turtles (Caretta caretta), which are vulnerable to reduced fitness and higher mortality when exposed to high temperatures in terrestrial nests (Reece et al. 2002; Fisher et al. 2014). I first determined
whether hsp expression differed between embryos from a temperate and a subtropical rookery. Secondly, I tested for and estimated the heritability of hsp expression, and the plasticity of hsp expression, in response to thermal stress. These results provide some key parameters needed for understanding whether long-lived reptiles could adapt to the unprecedented pace of contemporary climate change.

4.3 Materials and Methods

This research was conducted in accordance with the University of Western Australia (UWA) Animal Ethics Committee (RA/100/3/1195) and conforms to relevant guidelines for the care of experimental animals. Euthanasia protocols follow the American Veterinary Medical Association guidelines for embryonic reptiles. Fieldwork was conducted under permits SF009051 and SF009392 issued by the Western Australian Department of Parks and Wildlife (DPaW).

4.3.1 Study species and sites

Caretta caretta is a globally distributed species that has a relatively narrow nesting range confined to temperate and sub-tropical latitudes (Pike 2014; Baldwin et al. 2003). In Western Australia there is a large temperate rookery at Turtle Bay on Dirk Hartog Island (DHI; 25.49827 °S, 112.98719 °E) in Shark Bay and a smaller sub-tropical rookery at Bungelup Beach (BB; 22.282331 °S, 113.831570 °E) in Cape Range National Park (Fig 4.1). These two rookeries, along with smaller island and mainland rookeries comprise a single genetic stock in Western Australia (Pacioni et al. 2012) and together are considered the world’s third largest nesting population of the species (Baldwin et al. 2003; Reinhold & Whiting 2014). Nest temperatures are on average 2-3 °C cooler at DHI relative to BB during the respective peaks of summer nesting, and are subject to greater diel fluctuations of temperatures throughout the nesting season (Trocini 2013; Woolgar et al. 2014). Embryos developing on these beaches are vulnerable to thermal stress as they periodically experience temperatures above 35°C (Woolgar et al. 2014) and lethal heating events (Trocini 2013). Nest temperatures on these beaches are likely to increase in concert with rising air temperatures under climate change (Woolgar et al. 2014).
Figure 4.1. Dirk Hartog Island (DHI) and Bungelup Beach (BB) are two range-edge *C. caretta* rookeries in Western Australia, isolated by approximately 520km of coastline (Reinhold & Whiting 2014) and 3 degrees of latitude. DHI is located within a temperate zone while BB is a located within a sub-tropical zone (Pike 2014). Map adapted from Trocini (2013).
4.3.2 Sample collection and incubation experiments

I collected 1200 *C. caretta* eggs from Turtle Bay on DHI (80 eggs from each of 15 clutches) during peak nesting in late January 2013. Similarly, I collected 80 *C. caretta* eggs from Bungelup Beach (20 eggs from each of 4 clutches) at the end of peak nesting in early January 2014. Eggs were collected and transported following published protocols (Chapter 2).

4.3.3 Incubation and heat shock experiments

Eggs were distributed among 1.5 L plastic containers half-filled with moist white sand; each contained a randomly selected egg from each female to limit clutch effects (DHI: 80 containers each with 15 eggs, BB: 20 containers each with 4 eggs). All eggs were held in one of two 500 L incubators (Steridium, Brisbane, QLD, Australia) at 29 °C (± 0.3 °C), which is the pivotal temperature that produces a balanced sex ratio (Woolgar et al. 2014) for this population and provides near optimum incubation conditions. Following a previously established protocol for inducing a heat shock gene response in *C. caretta*, embryos were incubated for 45 days at 29 °C, and then either transferred to an incubator at 36 °C for 3 hrs (heat shock treatment), or else moved to an incubator at 29 °C for 3 hrs (procedural control) (Chapter 2).

4.3.4 Tissue collection and gene expression assays

After a one-hour cool down period [14], embryos were removed from the egg, weighed, and given a lethal injection of MS-222 (50 mg/kg) (Conroy et al. 2009; Chapter 2) followed by decapitation to ensure death. I selected cardiac tissue for gene expression analysis, as it increases expression of *hsp* in response to heat stress (Chang et al. 2000; Li et al. 2012; Stecyk et al. 2012; Chapter 2). Whole heart was isolated and cut in half through the aorta, atria, and ventricle. Each half was immediately stored at –80 °C until RNA extraction. Paternity data from a concurrent study (Chapter 3) was used as criterion for selecting embryos from each family (mother-father pair) for gene expression assays. For each family, expression assays were performed on 3-5 offspring in each of the procedural control and heat shock treatments. Total RNA was extracted from each half of cardiac tissue, and purity and quantity of RNA were measured following previously published protocols (Chapter 2). Gene expression assays
were run in 10 μL reactions with 1 μL cDNA template, and 0.2 μM of each forward and reverse primer, using previously designed primers for 18s (internal control), hsp60, hsp70 and hsp90 for C. caretta (Chapter 2).

4.3.5 Statistical analyses

Cycle Threshold (Ct) values (the number of PCR cycles required for the fluorescence signal to cross a threshold line) obtained from real-time PCR were converted to relative gene expression values ΔCt and ΔΔCt using the methods described in similar studies (Livak & Schmittgen 2001; Chapter 2). A mean ΔCt value, which is the difference between the mean Ct value of the target gene (hsp60, hsp70 and hsp90) and the mean of the control (18s) gene, were calculated for each embryo in the procedural control and heat shock treatments. The mean ΔCt values for the procedural control samples represent baseline expression levels, whereas the mean ΔCt values for the heat shock treatment samples represent expression levels under thermal stress. The ΔΔCt values, which measure the change in relative gene expression between treatments, were calculated by subtracting the mean ΔCt value for the control samples for each clutch from the mean ΔCt value for each embryo in the heat shock treatment. These values represent the level of plasticity (up- or down-regulation) in gene expression in response to the heat shock treatment.

A linear mixed-effects model was used to estimate variance components between and within rookeries. The model included rookery and clutch nested within rookery as random factors. Comparing total variances explained by the full model with a model having one factor removed tested the significance of each level in the analysis. Variance components were calculated using REML, and the VarCorr function in the R package nlme (Pinheiro et al. 2014). All ΔCt and ΔΔCt values were log-transformed with $2^{-X}$, where X is the mean ΔCt or mean ΔΔCt value, prior to analysis.

The heritabilities of ΔCt values for hsp60, hsp70, and hsp90 were estimated using an ‘animal model’ in ASReml 3.0 (Gilmour et al. 1995; Gilmour et al. 2009). The animal model is a linear mixed model used for quantitative genetic analyses, which in this study can be used to estimate additive genetic variance.
and maternal effects, even when some pedigree information is absent (Kruuk 2004). A pedigree for these analyses was generated using available paternity information for each offspring (Chapter 3). Separate animal models were fitted to each treatment (control and heat shock), with rookery, offspring identity, and dam as random factors. The term ‘dam’ is the maternal identity and estimates the genetic and environmental variance that is common to different mothers in the data set. Rookery and offspring identity measure genetic variance between rookeries and individual offspring, respectively. When the only pedigree term in the best-fitting model was dam, broad-sense heritability was estimated (Falconer & Mackay 1996). However, when both dam and offspring were included in the model, narrow-sense heritability was estimated. Narrow-sense heritability is of primary interest because it measures the extent to which phenotypes are determined by genes inherited from both parents, and is hence directly related to the ability of a population to respond to selection (Falconer & Mackay 1996).

Heritability of expression across treatments (phenotypic plasticity) was also estimated using the mean ΔΔCt values for hsp60, hsp70, and hsp90. Again, animal models were fit to the data using the same parameters. For both the within treatment (ΔCt) and across treatment (ΔΔCt) estimates REML Likelihood-ratio tests (REML LRT; Gilmour et al. 2009) were used to find the best fitting model, by starting with a fully saturated model and then systematically reducing the number of parameters.

A bivariate animal model was constructed to test for a genetic correlation between levels of gene expression in the two different treatments, but models failed to converge. Consequently, Spearman’s rank correlation was used to analyse whether, at the clutch level, the rank order of mean expression was correlated between treatments for each gene, and if mean expression was correlated between genes for each treatment.
4.4 Results and Discussion

4.4.1 **Clutch is the most important component of variation in baseline levels and increased levels of expression for hsp60, hsp70, and hsp90**

I detected significant variation in the *hsp* expression in response to heat stress. For both the procedural control (29 °C, 3 h) and the heat shock (36 °C, 3 h) treatments, the variance components analysis revealed no significant differences in expression between rookeries (Table 4.1). However, there was a significant proportion of variation in *hsp* expression among clutches within rookeries for all target genes (Table 4.1). Approximately 30-50% of all variation in *hsp* expression was due to clutch effects. This suggests that the phenotypic variance explained by clutch may be due to the genetic constitution of the offspring, as well as maternal effects such as yolk quality or the thermal environment during early embryogenesis (e.g. the environment in utero immediately prior to oviposition) (Shine et al. 1997). Female *C. caretta* have been shown to alternate ‘active and stay warm’ and ‘passive and stay cool’ thermoregulation strategies to optimise reproductive output (Schofield et al. 2009; Fossette et al. 2012). While any flow-on effects of thermoregulation during embryogenesis are yet to be documented in sea turtles, thermoregulatory behaviours in oviparous lizards (*Bassiana duperreyi*) can have significant effects on hatchling phenotypes (Shine 1995).
Table 4.1. Results from the variance components analysis of relative gene expression within treatments (ΔCt) and across treatments (ΔΔCt).

<table>
<thead>
<tr>
<th></th>
<th>hsp60</th>
<th></th>
<th>hsp70</th>
<th></th>
<th>hsp90</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total variance</td>
<td>P</td>
<td>% of total variance</td>
<td>P</td>
<td>% of total variance</td>
<td>P</td>
</tr>
<tr>
<td>ΔCt 29 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>22.7</td>
<td>0.215</td>
<td>0.0</td>
<td>0.999</td>
<td>0.0</td>
<td>0.999</td>
</tr>
<tr>
<td>Clutch</td>
<td><strong>33.3</strong></td>
<td>&lt;0.001</td>
<td><strong>28.0</strong></td>
<td><strong>0.002</strong></td>
<td><strong>23.0</strong></td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>ΔCt 36 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>14.6</td>
<td>0.446</td>
<td>3.2</td>
<td>0.792</td>
<td>28.6</td>
<td>0.143</td>
</tr>
<tr>
<td>Clutch</td>
<td><strong>48.6</strong></td>
<td>&lt;0.001</td>
<td><strong>30.4</strong></td>
<td>&lt;0.001</td>
<td><strong>33.9</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ΔΔCt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>21.3</td>
<td>0.264</td>
<td>2.9</td>
<td>0.683</td>
<td><strong>30.4</strong></td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>Clutch</td>
<td><strong>39.3</strong></td>
<td>&lt;0.001</td>
<td>4.5</td>
<td>0.442</td>
<td><strong>11.8</strong></td>
<td><strong>0.041</strong></td>
</tr>
</tbody>
</table>

Significant variance components at the $P < 0.05$ level are highlighted in **bold** text.

4.4.2 There is geographic variation in the plasticity of expression for hsp90 in response to heat shock, but not in hsp60 and hsp70

Gene expression assays were conducted on 14 clutches from a temperate rookery (DHI: offspring $N = 78$) and four clutches from a sub-tropical rookery (BB: offspring $N = 18$). All offspring increased expression for all target genes in response to an acute heat stress, as expected (Chapter 2). Expression of hsp90 increased 50.8-fold in embryos from the temperate rookery in response to the heat shock, and 19.3-fold in embryos from the sub-tropical rookery (Fig 4.2). These fold change differences were reflected by the significant proportion of total variance in ΔΔCt between rookeries for hsp90 (Table 4.1). Large fold-changes were also evident for hsp60 and hsp70 (Fig 4.2), but the differences between rookeries for these genes were non-significant (Table 4.1). As found in
the within-treatment analyses for all genes, there was a significant proportion of variance in relative gene expression between clutches within rookeries for \textit{hsp60} and \textit{hsp90}.

\textbf{Figure 4.2.} Expression levels of \textit{hsp60}, \textit{hsp70}, and \textit{hsp90} by \textit{C. caretta} embryos from a temperate (DHI, hatched bars; \( n = 129-130 \) per gene) and sub-tropical (BB, grey bars; \( n = 30 \) per gene) rookery in response to an acute heat shock. All mRNA data are normalised to the internal control 18s (values are clutch mean ± S.E.M.; \( N = 18 \)). Significant differences (\( P < 0.05 \)) between rookeries denoted with an asterisk.

Higher relative gene expression in \textit{hsp90} in the sub-tropical rookery is consistent with the nest temperature differences between the two study sites (Trocini 2013; Woolgar et al. 2014), and suggests that embryos developing at these beaches may be locally adapted. On Ascension Island in the South Atlantic Ocean, the black sand of Northeast Bay (NEB) is on average 2.6 °C warmer than the white sand of Long Beach (LB), and a common garden
experiment revealed that offspring of green sea turtles (*Chelonia mydas*) nesting on these beaches were locally adapted on a fine spatial scale (Weber et al. 2012). Although offspring from the NEB rookery exhibited greater tolerance to a warmer and wider range of temperatures, hatching success was significantly lower than at the LB rookery, perhaps reflecting embryonic thermotolerance failing to evolve with climate change (Weber et al. 2012). In this study, stronger evidence for local adaptation would require replication at the rookery level and knowledge of the genetic relationships between nesting females (Meier et al. 2014). Nevertheless, that *hsp90* has significant heritable plasticity in expression may provide *C. caretta* embryos with a mechanism for increasing their thermotolerance as a response to climate change. Further, the degree to which plasticity allows mean phenotypes to track changes in the thermal environment will determine whether nesting populations evolve to tolerate changes in nest temperature (Ghalambor et al. 2007).

### 4.4.3 Variation in hsp expression is heritable in *C. caretta*

The animal models used to estimate heritability (see Materials and Methods) considered the effects of rookery, offspring identity, and dam on *hsp* expression. Rookery had no significant effect on phenotype and was thereafter excluded from all models. The REML LRT showed that offspring was the best fitting pedigree factor in the animal model in *hsp70*, and so narrow-sense heritability was estimated for this gene. For *hsp60*, narrow-sense heritability was estimable in the heat shock treatment at 36 °C, but not in the procedural control treatment at 29 °C (Table 4.2). The estimates of narrow-sense heritability for expression of *hsp60* and *hsp70* were high relative to those expected of physiological traits for ectotherms (Mousseau & Roff 1987). In general, physiological traits (e.g. O\textsubscript{2} consumption, resistance to heat stress) have mid-level heritabilities (~0.33), similar to those for behavioural traits (e.g. alarm reaction, activity level; ~0.30), greater than those for life history traits (e.g. fecundity, survival; ~0.26), but much lower than those for morphological traits (e.g. body size, wing size; ~0.46) (Mousseau & Roff 1987).
Table 4.2. Heritability estimates for each heat shock gene within each treatment (ΔCt) and across treatments (ΔΔCt).

<table>
<thead>
<tr>
<th></th>
<th>hsp60</th>
<th>hsp70</th>
<th>hsp90</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCt 29 °C</td>
<td>0.51 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.23</td>
<td>0.32 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔCt 36 °C</td>
<td>0.79 ± 0.28</td>
<td>0.75 ± 0.27</td>
<td>0.21 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔΔCt</td>
<td>0.38 ± 0.21</td>
<td>0.20 ± 0.10</td>
<td>0.37 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Heritability values are listed with ± SE; all are narrow-sense heritabilities, with the exception of values denoted with a superscript, which are broad-sense heritabilities.

<sup>a</sup> Broad sense heritability (i.e. ‘dam’) was the only pedigree factor included in the best fitting model. Significance of heritability estimates determined using REML Likelihood Ratio tests (Gilmour et al. 2009) are shown in **bold**.

The heritability estimates of the plasticity in expression of the <i>hsp</i> genes were lower than the relative expression within treatments, but were nevertheless significant (Table 4.2). This is not unexpected, as heritability estimates for plasticity of a trait are typically lower than the heritability of the trait itself (reviewed by Scheiner & Lyman 1989 and Scheiner 1993). Taken together, these results show that populations have the potential to adapt through selection to increase the baseline expression levels of <i>hsps</i> (ΔCt) and also through selection on plasticity (ΔΔCt).

The differences in heritability estimates between relative expression and plastic expression could be due to a cost-benefit trade off (Scheiner 1993; Feder et al. 1997) in which expression of <i>hsp60</i> and <i>hsp70</i> are regulated to minimise any detrimental effects of overexpression (Hoffmann et al. 2003). Overexpression of <i>hsp70</i> compromises the fitness and survival of <i>Drosophila</i> larvae (Feder et al. 1997), but the rate at which developmental temperatures rise may affect how relative expression of <i>hsp70</i> evolves in natural populations (Krebs & Feder 1997). Hence it is possible that moderate increases in relative expression of
hsp60 and hsp70 (Fig 4.2) produce a short-term thermotolerance response in embryonic C. caretta, but levels of expression are tightly controlled to ensure embryonic survival.

For hsp90 only broad-sense heritability could be estimated. Relative to hsp60 and hsp70, the low but significant heritability estimates for hsp90 were similar to estimates for other physiological traits in other species [Mousseau & Roff 1987; Scheiner & Lyman 1989; Scheiner 1993; Krebs & Feder 1997]. The broad-sense heritabilities I estimated do not exclude the possibility of additive genetic variance in the expression of these genes, but do suggest that there may be more maternal, environmental or dominance effects at play. Clearly, model species such as Drosophila would be more amenable to separating these effects. However, as the heritability of relative expression of hsp90 has not been previously estimated in reptiles, future studies might examine whether increased expression levels of this hsp are consistent across clutches of individual females. For example, sampling from the multiple clutches laid by females within a single season (Miller 1997) could reveal if the heritability of plasticity in expression of hsp90 is characterised more by maternal or environmental effects.

Relative gene expression in the heat shock treatment was positively correlated with relative gene expression in the procedural control treatment for hsp60 (Spearman rank correlation coefficient $r_s = 0.911$, $P = <0.001$), hsp70 ($r_s = 0.624$, $P = <0.05$), and hsp90 ($r_s = 0.515$, $P = <0.05$). Thus, clutches that express highly in the control treatment also express highly in the heat shock treatment. These correlations suggest genes that increase expression in one thermal environment will also increase expression in another (e.g. Flatt et al. 2001; Groothuis et al. 2002).

The basal expression levels of different genes within environments were not correlated in the control treatment for hsp60 and hsp70 (Spearman rank correlation coefficient $r_s = 0.385$, $P = 0.114$), hsp60 and hsp90 (Spearman rank correlation coefficient $r_s = 0.259$, $P = 0.300$), or hsp70 and hsp90 (Spearman rank correlation coefficient $r_s = 0.424$, $P = 0.080$). In the heat shock treatment,
expression levels for \textit{hsp60} and \textit{hsp70} were correlated (Spearman rank correlation coefficient $r_s = 0.488$, $P = 0.040$). Increased expression levels \textit{hsp60} and \textit{hsp90}, were not correlated, although this was marginally non-significant (Spearman rank correlation coefficient $r_s = 0.451$, $P = 0.060$). Finally, increased expression levels of \textit{hsp70} and \textit{hsp90} were not correlated (Spearman rank correlation coefficient $r_s = 0.152$, $P = 0.550$). These correlations do not suggest that selection for increased expression in one gene may give rise to increased expression in other genes. However, the correlation between increased expression levels of \textit{hsp60} and \textit{hsp70} in the heat shock treatment implies that an increase in expression of one gene results in an increase of expression in both genes (Groothuis et al. 2002).

4.5 Conclusions

This is the first study to examine geographic and genetic variation in a physiological response to heat stress in sea turtle embryos, and the heritability of that response at the molecular level for any reptile species. Adaptation to changing environments requires physiological traits to be heritable, and I have shown heritability of expression of heat shock genes under both control and heat stress conditions. Moreover, I have shown that there is heritability in the plasticity of the expression of these heat shock genes. While expression levels of \textit{hsp60} and \textit{hsp70} were attributed to both maternal and additive effects, expression levels of \textit{hsp90} were characterised by maternal and environmental effects. Additionally, I found strong correlations between expression levels of all target genes and incubation environments, suggesting that an elevated level of baseline expression results in relatively higher expression under thermal stress. Taken altogether, this study presents the first evidence that sea turtles could possess a molecular mechanism for adapting to some degree of climate change.
4.6 References


Trocini S. 2013 *Health assessment and hatching success of two Western Australian loggerhead turtle (Caretta caretta) populations*. PhD thesis, Murdoch University, Perth.


Chapter 5: Heat shock gene expression in flatback turtles (Natator depressus) and loggerhead turtles (Caretta caretta)

5.1 Abstract

Embryonic sea turtles develop in terrestrial nests and are unable to behaviourally buffer themselves against increasing beach temperatures, so they must rely on physiological means to respond to environmental changes. There is evidence that loggerhead turtle embryos (Caretta caretta) can moderate thermal stress through expression of heat shock protein genes. However, in general, the thermotolerance of developing sea turtles is poorly understood. This study investigated the expression of heat shock genes in flatback turtles (Natator depressus). Embryos from two populations were subjected to an acute thermal stress of 36 °C for 3 hours and changes in expression of hsp60, hsp70, and hsp90 were measured by quantitative real-time PCR. There were no significant effects of rookery or clutch on baseline expression levels or in plasticity of expression for any of the target genes. The N. depressus data were then compared to existing data for loggerhead turtles, C. caretta, and similarly, no significant effects of species or rookery on baseline or plastic expression
levels of any target gene were detected. Potentially, nest temperatures experienced during development by both species are not very different and hence their responses to a heat shock at the molecular level are similar. But, more realistically, it is likely that statistical power was too low to detect any significant species or rookery effects.

5.2 Introduction

5.2.1 Thermal stress and thermotolerance of sea turtle embryos

Extreme environmental changes are a major threat to biodiversity (Bellard et al. 2012) by negatively impacting species physiology and thermal limits (Tewksbury et al. 2008; Rezende et al. 2011). Ectotherms are particularly sensitive to temperature changes (Bellard et al. 2012), as thermal stress can have devastating effects on locomotion, growth, reproduction, and sex determination (Tewksbury et al. 2008). Species may respond at the physiological level by tolerating warmer or drier climates, or by modifying diets, energy budgets, and activity levels (Bellard et al. 2012). Additionally, phenotypic plasticity of these responses and genetic adaptation could allow ectotherms to persist in their current environments despite climate change (Urban et al. 2014). Even though temperature increases may reduce fitness in some populations, it could cause strong selection on climate-related traits in others (Urban et al. 2014).

Reptiles such as sea turtles are vulnerable to anthropogenically-forced climate change (Howard et al. 2014), as their complex life cycle requires the terrestrial incubation of offspring. Embryos must develop on land to exchange water and oxygen through the amniotic egg to successfully grow into hatchlings (Ewert 1985; Ackerman & Lott 2005). Fixed in their nest environment, developing embryos are susceptible to mortality from extreme changes in incubation temperature (Valverde et al. 2010). Unlike the embryos of live-bearing species, the immobile embryos of egg-laying species have a limited capacity to ‘behaviourally buffer’ themselves against increasing temperatures (Du et al. 2011; Telemeco et al. 2013, Zhao et al. 2013; Pike 2014). However, there is
evidence that turtle embryos can use physiological mechanisms to respond to increased incubation temperatures (Gao et al. 2014; Chapters 2 and 4).

Recent studies have shown that embryonic Chinese-soft shelled turtles (*Pelodiscus sinensis*) and loggerhead turtles (*Caretta caretta*) increase expression of heat shock protein genes in response to heat stress (Gao et al. 2014; Chapters 2 and 4). For *C. caretta*, the plastic expression of *hsp90* varied across their geographic range in Western Australia (Chapter 4). Furthermore, increased expression of heat shock protein genes (*hsp9s*) is heritable, and thus this thermotolerance mechanism provides a basis for adaptive evolution (Chapter 4). However, it is crucial to understand how this thermal stress response varies between reptile species, as some species may be less resilient than others to climate change (Bellard et al. 2012; Urban et al. 2014).

This study investigated the variation in heat shock gene expression in flatback sea turtles (*Natator depressus*, Garman 1880), a species endemic to tropical Australia (Limpus 2007) and susceptible to extreme incubation temperatures (Pendoley et al. 2014; van Lohuizen 2014). Contrary to the pelagic life cycle of loggerhead turtle hatchling and juveniles, the life cycle of flatback turtles is characterised by near-shore development of hatchlings and juveniles until sexual maturity (Walker & Parmenter 1990; Miller 1997). As successful reproduction is dependent on near-shore habitats, understanding the resilience of this species to environmental change is critical.

### 5.2.2 Study species

*Natator. depressus* are the most poorly researched sea turtle species, listed as ‘Data Deficient’ by the IUCN Red List of Endangered Species (IUCN 2014) and ‘Endangered’ by the Environment Protection and Biodiversity Conservation Act of 1999. The juvenile and adult phases are generally spent in shallow turbid inshore waters (Limpus et al. 1983; Walker & Parmenter 1990; Bjorndal 1996), thus their life cycle is closely tied to nesting grounds (Limpus 2007). *Natator depressus* nest along the Australian continental shelf (Limpus 2007) in tropical latitudes below -24.8 °S (Pike 2013).
In Western Australia, nesting extends from Barrow Island, an offshore rookery near the southern limit of the range, through the Pilbara coast and further north through the Kimberley region (Limpus 2007), with other significant offshore rookeries near the Mundabullangana coast (Limpus 2007; Box 2010). Nesting of the Western Australian Northwest Shelf population occurs predominantly in summer months (Pendoley 2005; Limpus 2007).

5.2.2 Aims of study

The first aim of this study was to describe the hsp gene expression profiles of *N. depressus* from two different rookeries. The second aim was to compare the hsp gene expression profile of *N. depressus* to the gene expression profile of *C. caretta*, which was characterised in a previous study (Chapter 4) and is from a different genera in the Cheloniidae family. Significant variation in heat shock gene (hsp) expression between the two species is expected because embryos of *N. depressus* develop on tropical beaches (Limpus 2007) compared to *C. caretta* embryos, which typically develop on temperate and sub-tropical beaches (Pike 2014). Tropical environments are relatively warmer and more stable, while temperate environments are relatively cooler and subject to greater daily fluctuations in temperature (Sheldon et al. 2011; Huey et al. 2012). Because *N. depressus* develop on tropical beaches, where the thermal environment is warm and stable, and *C. caretta* develop on temperate or sub-tropical beaches, where the thermal environment is cooler and variable, it was predicted that: 1) *N. depressus* would have a higher baseline level of heat shock gene expression compared to *C. caretta*, and 2) *N. depressus* would have lower plasticity in heat shock gene expression compared to *C. caretta*. It was hypothesised that interspecific variation in hsp expression would be found because species from warm stable environments are generally near their thermal limits, and therefore may have higher levels of baseline hsp expression (Buckley et al. 2001; Tomanek 2010). Lower plasticity in *N. depressus* was predicted because higher baseline expression may weaken their ability to increase hsp expression following a heat shock (Buckley et al. 2001; Tomanek & Somero 2002).
5.3 Materials and Methods

5.3.1 Study sites

*Natator depressus* eggs were collected from two rookeries near the southern and central regions of the summer nesting range in Western Australia. The Barrow Island rookery (BI) lies approximately 60 km from the mainland coast and comprises of seven high-density nesting beaches where approximately 4,000 females nest annually (Pendoley et al. 2014). Cemetery Beach (CB) is a much smaller mainland rookery, at 1 km in length, and is the town beach for Port Hedland, with approximately 350 nesting females per season (Pendoley et al. 2014). Mean nest temperatures (°C) during the season are significantly higher at Cemetery Beach (33.8 ± 0.6) than at Barrow Island (31.7 ± 0.6; Fig. 5.3.1) (Pendoley et al. 2014), and may be responsible for significantly lower hatch success rates at this rookery (57.3 % compared to BI at ~85 %) (Foster 2008; Pendoley et al. 2014). Because of this, the Cemetery Beach rookery is considered to be a ‘hot spot’ beach and has the lowest hatch success on record for *N. depressus* (Pendoley et al. 2014).
Figure 5.3.1. Mean nest temperatures during the nesting season at two flatback rookeries (denoted with yellow stars) and at two loggerhead rookeries (denoted with blue stars). Peak nesting occurs in December and January for both species (Baldwin et al. 2003; Pendoley et al. 2014). Mean nest temperatures are significantly different between Barrow Island and Cemetery Beach (Pendoley et al. 2014) and between Turtle Bay and Bungelup Beach (Trocini 2013).

5.3.2 Sample collection and incubation setup

Eggs were collected from Cemetery Beach in Port Hedland during the peak-nesting season in December 2013 and from Yacht Club North Beach on Barrow Island during the peak-nesting season in January 2014. Ten clutches were collected from BI and two clutches at BB during oviposition, uniquely labelled for identification, and then packed in chilled sand for transportation off the beach. Within three hours of collection, eggs were removed to a field station and
packed in moist vermiculite into an Engel refrigerator (ENGEL MT45F-S, Australia) set at 7-10 °C to suspend embryonic development (Harry & Limpus 1989). Eggs were transported by air to Perth Domestic Airport and then driven to the University of Western Australia for incubation within 72-hours of oviposition.

Eggs were distributed among 1.5 L plastic containers half filled with moist sand and gradually warmed to room temperature in the laboratory; each contained a randomly selected egg from each female to limit clutch effects (BI: 20 containers of 10 eggs; CB: 20 containers of 2 eggs). Eggs were maintained in two 500 L incubators (Steridium, Brisbane, Australia) at 29.0 °C (± 0.3 °C) until the heat shock was applied. This temperature was chosen because it is the pivotal temperature for sex determination in *C. caretta* embryos from Western Australia (Woolgar et al. 2013), and because it was used in a previous study (Chapter 4), consistency across experimental conditions needed to be maintained. Pivotal temperatures (Tpiv) produce an equal male-female sex ratio (Mrosovsky 1994) and are generally viewed as optimal for embryonic development and viability (Ackerman & Lott 2005; Drake & Spotila 2002). The pivotal temperature for *N. depressus* embryos has been estimated at 29.3 °C and 29.5 °C from Mon Repos in Queensland (Limpus 2007 and Hewavisenthi & Parmenter 2000, respectively), 29.4 °C from Cape Dommett near the Western Australia and Northern Territory border (Stubbs et al. 2014), and 30.1 °C from the Dampier Archipelago (Box 2010).

De-ionized water was sprayed every 2 days to maintain humidity, and sand temperatures were monitored with Thermocron® loggers (Thermocron® iButton DS1921H, Thermodata, Brisbane, QLD; accuracy ± 0.125 °C, precision, ± 0.125 °C). Throughout incubation, containers were systematically moved between shelves and incubators to minimise any effects of subtle temperature variation within and between the incubators.

5.3.3 Heat shock experiments and tissue collection
A development rate function fitted for the Pilbara population of *N. depressus* (Box 2010) was used to estimate when embryos were approximately 85 %
developed (e.g. Chapter 4). This was to ensure embryos from both *C. caretta* and *N. depressus* were the same approximate age/stage of development for comparison of their gene expression profiles.

Embryos of *N. depressus* assigned to the heat shock treatment received a heat shock at Day 48 of incubation (86 ± 2 % of total development time to hatching). At Day 48, embryos selected at random for the heat shock treatment were moved from their incubator set at 29 °C to another incubator set at 36 °C for 3 hours, then returned to their original incubator at 29 °C for a one hour ‘cool-down’ period prior to euthanasia (Chapters 2 and 4). Embryos in the procedural control treatment remained at 29 °C until the time of sacrifice. However, to simulate the same movement and level of disturbance experienced by the heat shock embryos, the procedural control embryos also followed a mock ‘heat-up, cool-down’. This was performed by moving boxes containing the procedural control embryos from one incubator set at 29 °C to another incubator also set at 29 °C.

Euthanasia was performed by decapitation without anaesthetic, as the anaesthetic used for *C. caretta* (Chapters 2 and 4) had proven ineffective on *N. depressus*. After the ‘cool-down’ period, embryos were removed from the egg, weighed, and euthanised by severing cervical vertebrae with surgical grade bone shears. Brain tissue was isolated and placed into RNALater (Life Technologies, Mulgrave, VIC, Australia) to be stored at -80 °C for later analysis. Heart tissue was also isolated, cut in half vertically through the aorta, atria, and ventricle. Each half was preserved in RNALater at -80 °C for duplicate RNA extraction and gene expression assays.

5.3.4 Gene expression assays

Gene expression assays were conducted on up to ten offspring chosen at random from each clutch (*n* = 5 procedural control, *n* = 5 heat shock), totalling 97 offspring from the BI rookery and 20 offspring from the CB rookery. Total RNA was extracted from each half of heart tissue with FavorPrep™ Total Tissue Mini RNA Kit (Fisher Biotec, Subiaco, WA, Australia). Purity and quantity of RNA from each extraction were measured with a Qubit® 2.0 Fluorometer
(Invitrogen, Eugene, OR, USA). Following the protocol described in Chapters 2 and 4, only samples with quantity greater than 22.3 ng per μL in at least one extraction were used for cDNA synthesis and gene expression assays with real-time quantitative PCR (qPCR). The extracted RNA was normalised to 200 ng per μL and converted to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) as per manufacturer’s instructions.

Gene expression assays were performed by quantitative real-time PCR. Primers for housekeeping gene 18s and heat shock genes for hsp60, hsp70, and hsp90 that were previously developed for C. caretta (Chapter 2) had cross-amplified for N. depressus. Assays were run on a StepOnePlus™ Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with iTaq Universal SYBR® Green Supermix (Bio-Rad Technologies, Gladesville, NSW, Australia) in 10 μL reactions with 1 μL cDNA template, and 0.2 μM final concentration each F and R primer. Cycling conditions were 40 cycles of 95 °C for 10 min, 95 °C for 10 s and 60 °C for 60 s, with a melt curve generated to monitor for primer degradation.

**5.3.5 Statistical analyses**

Statistical analyses were performed on mean ΔCt and ΔΔCt values from each sample using the 2^−ΔΔCt method described in Livak & Schmittgen (2001). Cycle Threshold (Ct) values (the number of PCR cycles required for the fluorescence signal to cross a threshold line) obtained from qPCR assays were converted to relative gene expression values ΔCt and ΔΔCt. A ΔCt value is the difference between the Ct value of the target gene (hsp60, hsp70 and hsp90) and the internal control (18s) gene; these were calculated for each of the procedural control and heat shock treatment samples.

Baseline expression levels were represented by the ΔCt values for the procedural control samples, and expression levels under acute heat stress were represented by the ΔCt values for the heat shock treatment samples. Relative gene expression between treatments was calculated with the ΔΔCt values by subtracting the mean ΔCt value for the control samples for each clutch from the ΔCt value of each of the heat shock samples. These ΔΔCt values represent the
level of plasticity (up- or down-regulation) in gene expression in response to the heat shock treatment (Chapter 4).

The fold change in expression for each target gene was quantified for \textit{C. caretta} in a previous study (Chapter 4), and the results are replicated in Figure 5.4.2 for ease of comparison with results for \textit{N. depressus}.

Linear mixed-effects models were used to estimate variance components for the within species (\textit{N. depressus}) and between species (\textit{N. depressus} and \textit{C. caretta}). The full model used for the within species analysis contained rookery and clutch (nested within rookery) as random factors. The full model for the between species analysis contained species, rookery (nested within species) and clutch (nested within rookery) as random factors. Comparing total variances explained by the full model with a model with one factor removed tested the significance of each level in both analyses. Variance components were calculated using REML, the \texttt{VarCorr} function in the R package \texttt{nlme} (Pinheiro et al. 2014). All $\Delta \text{Ct}$ and $\Delta \Delta \text{Ct}$ values were log-transformed with $2^{-X}$ (where $X$ is the $\Delta \text{Ct}$ or $\Delta \Delta \text{Ct}$ value) prior to analysis.

To estimate the likelihood of detecting a significant rookery effect in the within species (\textit{N. depressus}) analyses, simulated data sets (100) were created with differences between rookery means ranging from 10 to 50%. The sample sizes and variances were the same as those in the actual data. Simulations were also used to estimate the likelihood of detecting a significant species effect in the combined species (\textit{N. depressus} and \textit{C. caretta}) analyses. As with the within species analyses, the simulated data were created using actual sample sizes and variances, with differences between species means ranging from 10 to 50%.
5.4 Results

5.4.1 No significant variation in the baseline levels of hsp expression in *N. depressus*

Gene expression assays were conducted on *N. depressus* embryos from 12 clutches from two tropical rookeries (BI: offspring $N = 185$; CB: offspring $N = 34$). There was no significant variation in expression levels for all target genes between rookeries or clutches within rookeries in the procedural control treatment ($29 \, ^\circ\, C, 3\, h$) or heat shock treatment ($36 \, ^\circ\, C, 3\, h$) (Table 5.4.1).

**Table 5.4.1.** Results from the variance components analysis of relative gene expression within treatments ($\Delta C_t$) and across treatments ($\Delta \Delta C_t$) for *N. depressus*.

<table>
<thead>
<tr>
<th></th>
<th>hsp60</th>
<th></th>
<th>hsp70</th>
<th></th>
<th>hsp90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total variance</td>
<td>$P$</td>
<td>% of total variance</td>
<td>$P$</td>
<td>% of total variance</td>
</tr>
<tr>
<td>$\Delta C_t , 29 , ^\circ, C$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>0.0</td>
<td>0.999</td>
<td>19.8</td>
<td>0.195</td>
<td>0.0</td>
</tr>
<tr>
<td>Clutch</td>
<td>17.9</td>
<td>0.098</td>
<td>1.8</td>
<td>0.824</td>
<td>1.8</td>
</tr>
<tr>
<td>$\Delta C_t , 36 , ^\circ, C$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>9.4</td>
<td>0.566</td>
<td>7.1</td>
<td>0.701</td>
<td>10.0</td>
</tr>
<tr>
<td>Clutch</td>
<td>10.8</td>
<td>0.281</td>
<td>18.6</td>
<td>0.089</td>
<td>5.7</td>
</tr>
<tr>
<td>$\Delta \Delta C_t$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rookery</td>
<td>0.0</td>
<td>0.999</td>
<td>0.0</td>
<td>0.999</td>
<td>0.0</td>
</tr>
<tr>
<td>Clutch</td>
<td>13.5</td>
<td>0.216</td>
<td>21.0</td>
<td>0.054</td>
<td>18.1</td>
</tr>
</tbody>
</table>

Significant variance components are shown in **bold** and are defined by a $P$-value <0.05.
5.4.2 No significant variation in the baseline levels of hsp expression between N. depressus and C. caretta

There was no significant variation in baseline expression levels or heat-shock expression levels for all target genes between species. However, the variance in expression of hsp90 in the heat shock treatment was marginally non-significant at the rookery level ($P = 0.070$, Table 5.4.2).

Table 5.4.2. Results from the variance components analysis of relative gene expression within treatments ($\Delta$Ct) and across treatments ($\Delta\Delta$Ct) for $N. depressus$ and $C. caretta$.

<table>
<thead>
<tr>
<th></th>
<th>hsp60</th>
<th>hsp70</th>
<th>hsp90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total variance</td>
<td>$P$</td>
<td>% of total variance</td>
</tr>
<tr>
<td>$\Delta$Ct 29 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>0.0</td>
<td>0.999</td>
<td>0.0</td>
</tr>
<tr>
<td>Rookery</td>
<td>15.2</td>
<td>0.111</td>
<td>1.3</td>
</tr>
<tr>
<td>Clutch</td>
<td>32.6</td>
<td>$&lt;0.001$</td>
<td>26.9</td>
</tr>
<tr>
<td>$\Delta$Ct 36 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>0.0</td>
<td>0.999</td>
<td>0.0</td>
</tr>
<tr>
<td>Rookery</td>
<td>7.1</td>
<td>0.391</td>
<td>2.2</td>
</tr>
<tr>
<td>Clutch</td>
<td>40.5</td>
<td>$&lt;0.001$</td>
<td>27.3</td>
</tr>
<tr>
<td>$\Delta\Delta$Ct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>0.0</td>
<td>0.999</td>
<td>2.9</td>
</tr>
<tr>
<td>Rookery</td>
<td>6.9</td>
<td>0.408</td>
<td>3.8</td>
</tr>
<tr>
<td>Clutch</td>
<td>38.4</td>
<td>$&lt;0.001$</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Significant variance components are shown in **bold** and are defined by a $P$-value <0.05.
5.4.3 No significant variation in plastic expression between the two species

For *N. depressus*, expression of *hsp60* increased 8.5-fold in embryos from Barrow Island, and 8.9-fold in embryos from Cemetery Beach (Fig. 5.4.1). For *hsp70*, expression levels in embryos from BI increased 16.4-fold, and in embryos from CB, expression increased 32.0-fold (Fig. 5.4.1). Variation in the plastic expression of *hsp70* was marginally non-significant between the rookeries (*P* = 0.054; Table 5.4.1). Increases in expression of *hsp90* in embryos from BI were 35.0-fold, and were 40.5-fold in embryos from CB (Fig. 5.4.1).

![Barrow Island and Cemetery Beach gene expression](image)

**Figure 5.4.1.** Relative gene expression in heart tissue of *N. depressus* embryos from Barrow Island (hatched bars; *n* = 90 per gene) and Cemetery Beach (grey bars; *n* = 16 per gene) following an acute heat shock. All mRNA data are normalised to the internal control *18s* (values are clutch mean ± S.E.M.; *N* = 12).

Large fold changes in expression of *hsp60*, *hsp70*, and *hsp90* by both *N. depressus* and *C. caretta* are evident when plotted together in Fig. 5.4.2. Although, expression levels of heat shock genes were not significantly different
at the species or rookery level, variation in plastic expression for \textit{hsp90} was marginally non-significant at the rookery level ($P = 0.068$, Table 5.4.2).

**Figure 5.4.2.** Relative gene expression in heart tissue of \textit{N. depressus} embryos from Barrow Island (hatched bars; $n = 90$ per gene) and Cemetery Beach (grey bars; $n = 16$ per gene), and \textit{C. caretta} embryos from Dirk Hartog Island (dotted bars; $n = 129-130$ per gene) and Bungelup Beach (dark grey bars; $n = 30$ per gene) following an acute heat shock. All mRNA data are normalised to the internal control \textit{18s} (values are clutch mean ± S.E.M.; $N = 30$).

**5.4.4 Power analyses**

Simulations to assess the statistical power of the within species analyses revealed a significant rookery effect would be detected in just 4% to 23% of cases (across all target genes) when there was a 10% difference in mean baseline gene expression between rookeries. These percentages increased to 45% to 97% when there was a 50% differences between rookery means. Similar results were obtained for the heat shock treatment and heat shock response (plasticity) analyses with significant effects detected in 0% to 45% of cases when there a 10% difference between means and 47 to 100% of cases when there was a 50% difference.
Simulations for the between species analyses also revealed there was limited statistical power. Across all genes and treatments, a significant species effect was detected in just 0% to 29% of cases when there was a 10% difference between means and 32% to 70% of cases when there was a 50% difference between means.

5.5 Discussion

Neither rookery, nor clutch contributed significant variation in hsp expression under control or heat shock conditions for *N. depressus*. These results suggest there was little variation between rookeries or between clutches within rookeries in this species. Most of the variation was within clutches, which may be attributed to both genetic and environmental effects. However, genetic relationships between embryos were unknown in this study so it can only be speculated to what proportion of variation in plasticity of expression was due to genetic effects or environmental effects. But since *N. depressus* exhibited large fold changes in hsp expression for at least two of the three target genes, the high plasticity might be sufficient for this species to produce phenotypic changes that could respond to the stresses of a new climate (Urban et al. 2014).

Nonetheless, it is possible that *N. depressus* sampled in this study may be locally adapted to their respective rookeries through mechanisms other than hsp expression, and this may explain the lack of geographic variation found in this study. Weber et al. (2012) concluded that green turtle hatchlings (*Chelonia mydas*) on Ascension Island were locally adapted to *in situ* conditions, however, mechanisms for this are unknown. Additionally, statistical power was very low for the within-species comparisons, so repeat sampling from Barrow Island and Cemetery Beach would need to be conducted to confidently conclude there are no differences between these rookeries. In addition, sampling from other *N. depressus* sites at lower latitudes could be conducted to compare gene expression profiles of embryos across a broader geographical range, which may reveal significant between-rookery effects.
The hypothesis that there would be significant differences in hsp expression profiles between species was not supported. There was no detectable significant variation in baseline expression levels or in plasticity between *N. depressus* and *C. caretta*. When the data was analysed overall, there were strong clutch effects but this was to be expected as there were significant clutch effects reported for *C. caretta* in Chapter 4. Therefore the clutch effects in this analysis could be reflecting the larger sample sizes in the between-species dataset than in the solitary *N. depressus* data set. There are several possible explanations for the lack of significant interspecific variation in hsp expression, three of which are discussed below.

Firstly, despite significant differences in mean nest temperatures recorded for these rookeries (Trocini 2013; Pendoley et al. 2014), nest temperatures across the sampling sites may not be sufficiently different to drive the evolution of differences in the responses of heat shock protein genes (Tomanek 2010). Baselines levels of expression were not significantly different between species, when it was expected that *N. depressus* would have naturally elevated baseline levels as a reflection of warmer *in situ* incubation conditions. This means if *N. depressus* and *C. caretta* embryos develop within environments that are not significantly variable from one another, then both species may be able to withstand a wide range of temperatures that allow for great levels of plasticity in hsp expression (Tomanek 2010). If *N. depressus* and *C. caretta* are exposed to longer periods of warming, this plasticity in expression may allow both *N. depressus* and *C. caretta* to increase hsp expression levels under greater temperature extremes, and ultimately adapt (Huey et al. 1999).

A second possible reason for lack of interspecific variation is that thermoregulatory behaviours by females during the nesting period may indirectly influence the thermotolerance of developing embryos. For example, studies show both female *C. caretta* at temperate nesting sites and *C. mydas* at tropical nesting sites exhibit ‘maternal thermophily’ by actively seek out warm water microhabitats at the start of the breeding season (Godley et al. 2002; Schofield et al. 2009; Weber et al. 2011; Fossette et al. 2012).
The peak in nesting for the mainland rookeries of Bungelup Beach and Cemetery Beach occur in December (Pendoley 2005; Chapter 4) and the peak in nesting for the island rookeries at Dirk Hartog Island and Barrow Island occur in January (Pendoley 2005; Trocini 2013; Chapter 4). Although mainland rookeries are known to be hotter than island rookeries (van Lohuizen 2014), sand temperatures in January tend to be greater than in December. Thus, temperatures during peak nesting could be quite similar across rookeries within species. While thermal differences between the rookery temperatures of the two species are likely (Fig. 5.3.1) maternal thermophily (Weber et al. 2011) that may explain the lack of significant differences in hsp expression within species may also act to reduce differences between species.

A third, and most likely, explanation for lack of interspecific variation in hsp expression is that statistical power was low in this study. Relative to the number of samples collected from Dirk Hartog Island (C. caretta) and Barrow Island (N. depressus), only small numbers of clutches could be collected from mainland rookeries (Bungelup Beach and Cemetery Beach). Additional sampling of clutches from Bungelup Beach and Cemetery Beach would increase statistical power. However, it will be difficult to collect more samples from these mainland rookeries because the nesting density is low (Trocini 2013; Pendoley et al. 2014).

In summary, despite differences in rookery temperatures between N. depressus and C. caretta, interspecific variation in expression of heat shock genes was not detected. Neither baseline expression levels nor plasticity of expression in hsp60, hsp70, and hsp90 varied between species for several plausible reasons. It is possible that there is little thermal variation in nest environments due to variation in the time of peak nesting, or that maternal thermoregulatory behaviour optimises reproductive output. Either way, statistical power was low in this study and further sampling would need to be conducted to test these hypotheses. Consequently, the results presented here should be considered preliminary and interpreted with caution.
5.6 References


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Projected climate warming in the latter half of the 21st century (IPCC 2014) poses a major threat to biodiversity (Bellard et al. 2012). Extreme climates can negatively impact species distributions, phenology, behaviour, physiology and reproduction (Howard et al. 2014; Urban et al. 2014). Changes in climate from the 20th century have already caused physiological responses in some species (Bellard et al. 2012). Ectotherms are particularly sensitive to environmental changes (Bellard et al. 2012), as increasing temperatures can negatively impact locomotion, growth, reproduction, and sex determination (Tewksbury et al. 2008). However, phenotypic plasticity and genetic adaptation could allow such species to persist in their current environments despite climate change (Urban et al. 2014).

To effectively understand species resilience to climate change, there is a need to understand the factors that determine resilience (Evans & Hofmann 2011; Sgrò et al. 2011; Fuentes et al. 2013). Further, there is a need to understand the relative importance of those factors in affecting the ability of species to
adjust to climate change (Fuentes et al. 2013; Howard et al. 2014). Sea turtles have persisted through dramatic changes in past climates (Pritchard 1997), and are thought to have adapted to past climates through spatial and temporal shifts in nesting areas (Hamann et al. 2007). Such behavioural plasticity may offer one of the best opportunities for adaptation in sea turtles (Hawkes et al. 2009; Schofield et al. 2009). However, sea turtles have very long generation times (Heppell et al. 2003) and extreme nest site fidelity (Lohmann et al. 2013), two life history traits that may prevent them from adapting quickly through evolved changes in behaviour. Therefore, phenotypic plastic responses at the physiological level may compensate when behavioural responses are not possible (Hoffmann & Sgrò 2011; Urban et al. 2014).

This thesis has demonstrated that sea turtle embryos exhibit a physiological response to thermal stress at three heat shock genes, \textit{hsp60}, \textit{hsp70}, and \textit{hsp90}. More importantly, high heritability in baseline expression and plasticity in expression of these genes suggests that adaptation to a warmer climate can act through selection on physiological traits that are associated with thermotolerance.

When given a thermal challenge (heat shock of 36 °C for 3 hours), embryos of \textit{Caretta caretta} significantly increased expression of \textit{hsp70} and \textit{hsp90} in heart and brain tissue (Chapter 2). Large fold changes in expression of these genes indicated a physiological response to the stress, and it was concluded that these two \textit{hsp}s are effective biomarkers for measuring thermal stress during embryonic development. These findings were novel for a non-traditional experimental model such as sea turtle, and notably, were consistent with the results of previous work on freshwater turtles (Stecyk et al. 2012; Gao et al. 2014). Expression levels of all three \textit{hsp}s were compared across two tissue types. Increases in expression of \textit{hsp60} were not significant for heart tissue across any of the experimental treatments but were significant for brain tissue in the most extreme treatment (36 °C, 3 h. The modest changes in expression of \textit{hsp60} in both tissue types indicated this \textit{hsp} is stable during development and therefore not an effective biomarker of thermal stress in embryonic sea turtles.
Nevertheless, expression changes in *hsp60* were analysed in all subsequent experiments to maintain consistency throughout the project.

The first step in understanding the capacity of the physiological response in sea turtles was to describe the phenotypic variation in *hsp* expression (Chapters 4 and 5). Then, to be able to estimate the genetic variation contributing to the plasticity of *hsp* expression, the frequency of multiple paternity must be uncovered to understand the genetic relationships between embryos within clutches (Chapter 3). The frequency of multiple paternity was determined for three Western Australian *C. caretta* populations by sampling from rookeries across the nesting range. Clutches sampled from Gnaraloo Bay exhibited the highest rates of multiple paternity at 86%, which were similar to estimates in Zakynthos Bay in Greece (Zbinden et al. 2007). At this site the bathymetry influences currents to funnel breeding males and females near the shore, creating a high rate of mate encounter. Gnaraloo Bay may be a male-producing rookery (Woolgar et al. 2013), and since the bay is positioned in the middle of a foraging corridor for this population, there is also a high probability of mate-encounter. Paternity estimates for the Dirk Hartog Island (DHI) and Bungelup Beach (BB) rookeries were 36% and 25%, respectively. The frequencies for these rookeries were consistent with estimates from the Northern Hemisphere and eastern Australia (Harry & Briscoe 1988; Bollmer et al. 1999; Moore and Ball 2002; Sakaoka et al. 2011). The paternity estimates for DHI and BB were used in the estimation of heritability in Chapter 4.

The baseline levels of *hsp* expression and plasticity in *hsp* expression detected in *C. caretta* in the preliminary heat shock experiment (Chapter 2) served as a platform for later studies of clutch-, population-, and species-level variation in the thermal stress response (Chapters 4 and 5). Geographic variation in *hsp* expression was investigated by sampling from rookeries at or near the edge of the Western Australian nesting populations of *C. caretta*. There was significant variation in *hsp* expression among clutches within rookeries for all target genes in both the procedural control treatment (baseline expression) and the heat shock treatment (36 °C, 3 h). Clutch also contributed a significant proportion of variation in the plastic response of *hsp60* and *hsp90*. Finally, there was also
significant variation in the plastic response of hsp90 at the rookery level, indicating geographic variation in response of this gene and possibly evidence of local adaptation (e.g. Weber et al. 2012).

Knowing that phenotypic variation in hsp expression was significantly different for one gene (hsp90) between rookeries, the genetic contribution to this variation was estimated. Heritability estimates of hsp60, hsp70, and hsp90 in both the procedural control and heat shock treatments were high compared to other physiological traits (Mousseau & Roff 1987), but the heritabilities of plasticity in expression of all target genes were relatively low compared to the heritabilities of expression in the control and heat shock temperatures. This was consistent with other studies that show lower heritabilities of a plastic response compared to the heritability of the trait itself (Scheiner & Lyman 1989; Scheiner 1993). Additionally, there were strong correlations between basal expression levels in the control treatment and increased expression levels in the heat shock treatment, suggesting that individuals that have high baseline expression also express highly in stressed conditions. Additionally, genetic correlations for increased expression of heat shock protein genes were found between hsp60 and hsp70. Together these results indicated that increased expression of heat shock genes is heritable in sea turtle embryos, and serves as platform on which selection can act for adaptation to some degree of climate warming.

Unlike the case for C. caretta embryos, variation in hsp expression was not significant at either the rookery or clutch level for N. depressus embryos. Furthermore, there was a lack of significant interspecific variation in hsp expression between C. caretta and N. depressus for any target gene under any treatment conditions. It is possible that despite the different mean nest temperatures at each rookery (Trocini 2013; Pendoley et al. 2014), the thermal variation in incubation environments may be modest and insufficient to elicit differences in hsp expression (Tomanek 2010). It is possible that thermoregulatory behaviour exhibited by females immediately prior to nesting optimises reproductive output and minimises thermal stress on developing embryos. However, the most likely explanation for the lack of interspecific variation in hsp expression is due to low statistical power. Further sampling to
increase the size of the dataset from the mainland rookeries may ultimately reveal significant interspecific differences.

6.1 Limitations of research

The key findings from this thesis have been discussed above. However, there were some limitations to the experiments I conducted, which are discussed below.

a. I designed an experimental protocol for inducing and measuring a heat shock gene response in embryonic sea turtles (Chapter 2). However, embryos were held at constant temperatures until the heat shock was applied, and the natural thermal variation of in situ nests should be considered (Horne et al. 2014).

b. During the initial experimental design phase for the gene expression assays (Chapter 2), the addition of protein quantification or protein expression assays was not considered. This was a criticism from several reviewers when the corresponding manuscript was submitted for publication. The lack of protein quantification data was justified in Chapter 2 by stating that only the transcription phase (mRNA) of protein building was considered in this study, and that protein concentration is generally quantified at the translation and degradation phase of protein building. Therefore the current practice of using mRNA concentration as a proxy for protein concentration is inaccurate. Knowledge of changes in protein concentration as a product of changes in hsp gene expression would be valuable for characterising thermotolerance in embryonic sea turtles.

c. In Chapter 3 paternity frequencies were estimated for hatchling samples that were previously collected for another study at Gnaraloo Bay. Maternal tissue was not required for this study and therefore was not collected. As a consequence, microsatellite genotypes obtained from the hatchling samples could not be matched against maternal genotypes to verify the multiple paternity estimates calculated from the GERUD 2.0 and COLONY 3.0
programs. Although these programs are robust and the results were reported with confidence, all future DNA work on embryos or hatchlings should include maternal genotypes for reference to ensure accuracy of paternity assignment.

d. Field work involving egg collection from nesting sea turtles is time-sensitive and sometimes costly and logistically difficult as some mainland rookeries can vary between 0-20 females per week of nesting (Riskas 2014). The small sample sizes of *C. caretta* from Bungelup Beach (Chapter 4) and *N. depressus* from Cemetery Beach (Chapter 5) may have attributed to low statistical power in the analyses. Non-significant results may otherwise have been significant if larger sample sizes had been able to be collected.

e. One of the original aims of the project was to determine rates of multiple paternity and to thereby estimate heritability of *hsp* expression for *N. depressus*. However, due to financial and time constraints, microsatellite genotyping for multiple paternity assignments of *N. depressus* could not be conducted. However, maternal and offspring has been stored and is available for any future analysis of paternity.

### 6.2 Directions for future research

To address the limitations of the research described above, and to advance the understanding of tolerance to extreme temperatures by embryonic sea turtles, the following future research directions are proposed:

a. As incubation conditions were held constant throughout the heat shock experiments, and embryos that develop *in situ* nests experience daily and seasonal fluctuations in temperature, future heat shock experiments investigating heat shock gene expression should be designed to simulate natural temperature fluctuations that embryos experience throughout incubation. Control conditions can still be held at Tpiv or similarly optimal temperatures for embryonic development, but those control conditions could fluctuate ± 0.1-0.5 °C on either side of that optimal temperature, on a timed cycle that embryos might experience in natural nests. Also, longer heat shock
periods which better simulate the stress of a heating event in a nest, or a higher ‘baseline’ temperature (e.g. 32 °C), can be applied to see if higher average incubation temperatures offer some protection against short periods of thermal stress (e.g. Hoffmann et al. 2003).

b. Future experiments should include quantifying the proteins produced by the genes as an additional measure of differential expression of the target genes. For example, instead of extracting RNA from both halves of the tissue for gene expression assays (Chapters 2 and 4), one half of the tissue can be reserved for protein expression/quantification.

c. The time course after a heat shock event could be evaluated to determine how long after such an event does hsp activity remain elevated (i.e. minutes, hours, days). This information would provide a better understanding of the limitations embryos may possess to mitigate the deleterious effects of thermal stress.

d. Since fieldwork of this nature is reliant on good weather (i.e. no possibility of cyclones) and high densities of nesting females, experimental designs should include plans for quick succession of multiple collection trips to maximise the number of samples that can be collected from lower density rookeries, such as occur on many mainland beaches.

e. Further laboratory work should be done to determine frequency of multiple paternity for *N. depressus*. If rates of multiple paternity are high, paternity information could be used for estimating the heritability of the *hsp* response in *N. depressus*, as it was done for *C. caretta*.

f. A link between heat shock gene expression and thermotolerance of embryos could be measured by calculating mortality rates when embryos are exposed to thermal stress. Then, conservation managers would be better informed as to which species could be vulnerable to population declines due to high embryonic mortality during heatwaves. This information could drive management strategies such as beach shading and re-vegetation (Hawkes et

6.3 Concluding remarks

With the onset of rapid environmental warming, higher incubation temperatures may have profound impacts on development and hatching success of sea turtle embryos. This research has demonstrated that embryonic *C. caretta* and *N. depressus* increase expression of heat shock genes, which may offer some resilience to thermal and other stressors, and that this response is plastic and highly heritable, providing a platform for adaptation. The limitations of plasticity in expression of heat shock genes requires further study given the current pace of environmental change.
6.4 References


Appendices: Published Papers
Increased expression of Hsp70 and Hsp90 mRNA as biomarkers of thermal stress in loggerhead turtle embryos (Caretta Caretta)

J.N. Tedeschi, W.J. Kennington, O. Berry, S. Whiting, M. Meekan, N.J. Mitchell

1. Introduction

The survival and viability of sea turtle embryos is dependent upon favourable nest temperatures throughout the incubation period. Consequently, future generations of sea turtles may be at risk from increasing nest temperatures due to climate change, but little is known about how embryos respond to heat stress. Heat shock genes are likely to be important in this process because they code for proteins that prevent cellular damage in response to environmental stressors. This study provides the first evidence of an expression response in the heat shock genes of embryos of loggerhead sea turtles (Caretta caretta) exposed to realistic and near-lethal temperatures (34°C and 36°C) for 1 or 3 hours. We investigated changes in Heat shock protein 60 (Hsp60), Hsp70, and Hsp90 mRNA in heart (n = 24) and brain tissue (n = 29) in response to heat stress. Under the most extreme treatment (36°C, 3 h), Hsp70 increased mRNA expression by a factor of 98.3 in heart tissue and 14.7 in brain tissue, while Hsp90 mRNA expression increased by a factor of 98.3 in heart tissue and 14.7 in brain tissue. Hence, both Hsp70 and Hsp90 are useful biomarkers for assessing heat stress in the late-stage embryos of sea turtles. The method we developed can be used as a platform for future studies on variation in the thermotolerance response from the clutch to population scale, and can help us anticipate the resilience of reptile embryos to extreme heating events.

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exposed to high temperatures (Hutter et al., 1994; Krivoruchko and Storey, 2010) and preserve the functioning of tissues until the stress period is relieved (Feder and Hofmann, 1999; Kulitz et al., 2005; Bapli et al., 2011).

Sea turtle embryos develop optimally within a narrow thermal range, and are vulnerable to mortality if temperatures exceed 35 °C (Ackerman, 1997; Davenport, 1997). Average incubation temperatures above 35 °C early in development rapidly increase the rate of metabolic activity and development, resulting in small embryos that hatch early (Davenport, 1997; Deeming, 2005). Temperatures above 35 °C late in development can reduce the fitness of hatchlings by altering swimming performance, causing disorientation and death from heat exhaustion (Blamires and Guinean, 2001; Drake and Spottia, 2002; Valverde et al., 2010). Climate change is expected to increase air temperatures by 3.5 °C in the coming decades (IPCC, 2007) and this will in turn increase the temperatures of nesting beaches. Little is known about the capacity of embryonic turtles to tolerate and adapt to warming nest environments (Fuentes et al., 2005; Weber et al., 2011). Thus, the physiological and molecular mechanisms underlying the thermotolerance response of sea turtle embryos need to be investigated (Hawkes et al., 2014; Pike, 2014).

Loggerhead sea turtles (Caretta caretta, Linnaeus 1758) are globally distributed (Baldwin et al., 2003) but have a relatively narrow nesting range compared to other sea turtles, mostly confined to the sub-tropics where beaches may experience significant variation in temperature during the nesting season (Pike, 2014). One of the world’s largest loggerhead rookeries occurs on Dirk Hartog Island in Shark Bay, Western Australia (Baldwin et al., 2003; Reintold and Whitting, 2014). Eggs buried on these beaches experience temperatures upwards of 35 °C (Woolgar et al., 2013) and occasional lethal heating events (Trocini, 2013) that are likely to increase in frequency as air temperatures rise under climate change. Here, through artificial incubation, we simulate in situ thermal stress and measure changes in mRNA levels of Hsp gene expression in response to that stress in C. caretta embryos. We hypothesise that the thermally sensitive Hsp60, Hsp70, and Hsp90 genes will increase mRNA expression in embryos as a response to heat stress, but more specifically that these genes will be good indicators of a thermotolerance response in this species. To test our hypothesis, we first developed a protocol designed to elicit and quantify a thermal response in embryos. Additionally, we compared the responses in whole heart and whole brain tissue, as these differ in heat-shock protein abundance and potentially have different gene expression profiles (Stecyk et al., 2012). Further, we assessed the impact of brief heat shocks on hatching success to determine whether our treatments were lethal, sub-lethal or beneficial. Finally, we discuss our findings as they relate to previous studies investigating thermotolerance responses in reptiles, as they are the first reported for the embryonic stage in the Cheloniidae lineage.

2. Materials and methods

2.1. Animals

This study was approved by the University of Western Australia Animal Ethics Committee (RA/100/3/1081) and conforms to relevant guidelines for the care of experimental animals. Euthanasia protocols follow the American Veterinary Medical Association guidelines for embryonic reptiles (Conroy et al., 2009; Leary et al., 2013).

2.2. Egg collection

Loggerhead turtle eggs were collected from Turtle Bay on Dirk Hartog Island in Western Australia (25°29′59.47″ S, 112°59′35.90″ E) in January 2012. Ninety-six eggs were collected from each of four clutches (total eggs = 384). They were excavated from the nest chamber after the female returned to the water, and were individually numbered, and packed in chilled sand for transportation off the beach. Within three hours of collection, eggs were re-packaged in moist vermiculite in a 40 L portable refrigerator (ENCEL MT45F-S, Australia) set at 7–10 °C to suspend embryonic development (Harry and Limps, 1989). Finally, chilled eggs were transported by boat and road to the University of Western Australia in Perth for incubation within a 72-hours of oviposition.

2.3. Incubation and monitoring

Eggs were gradually warmed to room temperature in the laboratory and distributed among 1.5 L plastic containers half filled with moist sand, producing 96 containers of 4 eggs, each containing one egg from each female. Two 500 L incubators (Sterifidium, Model e500, Brisbane, QLD, Australia) were set at the pivotal temperature of 29.0 °C for sex determination in this population (Woolgar et al., 2013). Pivotal temperatures are viewed as optimal temperatures for embryonic development and viability (Ackerman and Lott, 2005; Drake and Spottia, 2002). Sand was sprayed with de-ionised water every 2 d to maintain humidity, and containers were systematically moved between shelves and incubators to minimise any effects of subtle temperature variation within and between the incubators.

Day 0 of incubation was the start point for calculating the time to each treatment, and to hatching. Loggerhead embryos are defined as being in the early stages of incubation from day 0 to day 25 and in the late stage of incubation from approximately day 45 up until hatching at around 58 days (Miller, 1985; Miller et al., 2003; Mrvoskovski, 1980; Yntema, 1968). All containers were incubated at an average of 29.3 °C ± 0.3 °C until day 25 or day 45 of incubation when they received a heat shock, except for two procedural control groups that remained at 29 °C. Embryos that were not sacrificed after the heat shock treatments were returned to their original incubator and incubated until hatching (Fig. 1).

2.4. Heat shock Treatments

Two procedural control (PC1 and PC2) and four heat shock treatments (T1, T2, T3, and T4) were designed to determine the best combination of parameters for inducing a response to thermal stress (Fig. 1). These treatments were chosen because the temperatures were 1 °C on either side of the lethal field temperature reported by Davenport (1997), and because shocks of 1 or 3 hours simulate realistic environmental fluctuations without inducing instant mortality. The temperature and duration of the shock that the embryos experienced was divided into two blocks of experiments (Fig. 1). Egg containers received heat shocks at either Day 25 or Day 45 of incubation (termed Day 25 or Day 45 embryos throughout). Eggs were further allocated to either be euthanised immediately, or left to hatch and hence were returned to the 29 °C incubator (approximately 50%) (Fig. 1). We predicted that the most extreme heat shock, treatment 4 (36–3 °C, 3 h), would create a thermally stressful environment for embryos and gene expression levels would greatly increase in response.

We determined the time taken for the ‘heat shock’ to be detected within the incubation medium using small temperature loggers (Thermocrorn5™ iButton DS1921H, Thermodata, Brisbane, QLD, Australia; accuracy ± 0.125 °C, precision ± 0.125 °C) buried amongst the eggs. Temperatures reached the set points of 34 °C.
and 36 °C after two hours. Thus, containers remained inside the incubators at the heat shock temperature for a total of three hours (2 h ramping + 1 h shock) or five hours (2 h ramping + 3 h shock), depending on the treatment group. The procedural control groups were moved to another 29 °C incubator for 1 or 3 hours to simulate the disturbance experienced by the heat shock groups.

After the heat shock the egg containers were returned to 29 °C for a one hour ‘cool-down’ period prior to euthanasia. This ‘heat-up, cool-down’ process follows similar protocols developed for flies, frogs, lizards, and freshwater turtles (see Krebs et al., 1998; Seensan et al., 2009; Ullasov et al., 1992; and Ramaglia and Buck, 2004). The procedural controls also followed a mock ‘heat-up, cool-down’ regime to simulate the same movement and disturbance conditions as embryos in the heat shock treatments.

### 2.5. Heat shock treatments and euthanasia of embryos and hatchlings

Hatching success after a heat shock treatment (or procedural control) was also investigated for both Day 25 and Day 45 embryos. Eggs randomly selected from each container for heat shocks or procedural controls on day 25 of incubation (n=96) were removed from 29 °C incubators and subjected to one of treatments T1, T2, T3, or T4. Half of these embryos (n=48) were euthanised after the heat shock by physical removal from the egg and dispatching the chorion-allantoic membrane to induce respiratory and circulatory death (Deeming, 2005). Once removed from the eggs, embryos were placed onto a glass petri dish and the head was removed with a sterile scalpel blade and stored in RNA Later (Life Technologies, Mulgrave, VIC, Australia) at –20 °C. The abdomen was stored at 4 °C in 100% ethanol for DNA extraction. The remaining 48 embryos were returned to the 29 °C incubator until hatching.

The same procedures were followed for eggs subjected to heat shocks or procedural controls on day 45 of incubation (n=96), except that euthanasia was achieved by physical removal from the egg and lethal injection by overdose of MS-222 into the coelom, following the protocol of Connery et al. (2009). This anaesthetic agent was used in preference to barbiturates such as sodium pentobarbital because the integrity of genetic material is maintained (Barreto et al., 2007; Palmisano et al., 2000). Once euthanasia was confirmed by an absence of papillary response, the cervical vertebrae were severed with surgical grade bone shears. The head was removed with sterile scissors and the skullcap was peeled back to expose the brain. The brain was removed with a sterile micro-spatula and placed into RNA Later to be stored at –80 °C. To access the heart, cuts were made down the coronal plane on either side of the plastron to peel away the pericardium. The heart was removed with the sterile surgical scissors and forceps and placed into RNA Later to be stored at –80 °C. The hatch date of embryos that continued incubation after the heat shock treatments or procedural controls was determined as the day that the shell first ruptured. Once hatchlings were fully emerged they were weighed and euthanised by intraperitoneal injection of a lethal dose of pentobarbital sodium following the methodology described in Woolgar et al. (2013).

### 2.6. Total RNA extraction and Quantification of Day 45 embryos

Gene expression analysis was conducted for Day 45 embryos only, as preliminary results indicated that sufficient quantities of RNA could not be extracted from Day 25 embryos. Total RNA was extracted from whole brain and heart in late embryos (FavorPrep™ Total Tissue Mini RNA Kit, Fisher Biotech, Subiaco, WA, Australia). Purity and quantity of RNA were measured with a Qubit™ 2.0 Fluorometer (Invitrogen, Eugene, OR, USA). RNA quality was evaluated by gel electrophoresis on a 1.5% Agarose gel and 1 × TAE Buffer. Only samples showing strong bands and quantity greater than 22.3 ng/μl were used for cDNA synthesis. A total of 24 heart samples and 29 brain samples were analysed for changes in gene expression.

### 2.7. RT-PCR primer design for 18 s, Hsp60, Hsp70, Hsp90

Previously described primers for the housekeeping gene 18 s (Hillis and Dixon, 1991) and heat shock protein gene Hsp60 (Kohno et al., 2010) were used to generate sequences for C. caretta. For Hsp70 and Hsp90 relevant GenBank sequences were aligned and primer pairs designed using Oligo v6.8 (Rychlik and Rhoads, 1989).

#### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number for Sequences for RT-PCR</th>
<th>Primers for qPCR</th>
<th>Annealing Temperature (°C)</th>
<th>Efficiency</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 s</td>
<td>KJ683738</td>
<td>F: GCTTATACATATGCGGACCGGAG 60.8</td>
<td>110.77%</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCCCGCGGAGTGTGACGGAC 60.4</td>
<td>103.15%</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td>KJ683735</td>
<td>F: TACGAGACGCTGAACGGCT 59.4</td>
<td>108.78%</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGCCTTGAAAGGATCCCTG 60.5</td>
<td>106.28%</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>Hsp70</td>
<td>KJ683736</td>
<td>F: TTCCGCTACAGCTTGACG 60.4</td>
<td>108.78%</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACCGCAAGATACAGC 60.0</td>
<td>106.28%</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>Hsp90</td>
<td>KJ683737</td>
<td>F: GGCAGTGGCGAAAGGGCAG 59.3</td>
<td>106.28%</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACACACAAAAGTACGAAC 60.3</td>
<td>106.28%</td>
<td>0.996</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. A factorial block design, sample sizes for each procedural control (PC1 and PC2) and heat shock treatment (T1, T2, T3, T4) at either Day 25 or 45 of incubation. Subsets of embryos were euthanised or left to hatch. Eggs were divided so that half of these embryos (n=48) were moved to another 29 °C incubator for 1 or 3 hours to simulate the disturbance experienced by the heat shock groups.

Table 1

RT-PCR sequences modified and designed for Caretta caretta-specific primer pairs for real-time PCR assays. Efficiencies and coefficients of determination are given for each primer pair.
2.8. Real-time PCR primer design and amplification of 18 s, Hsp60, Hsp70, Hsp90

A SYBR/intercalating dye was chosen to measure mRNA gene expression. Primers were designed to amplify an approximately 150 bp region of the genes. 18 s, Hsp60, Hsp70 and Hsp90 were sequenced by the Australian Genome Research Facility (AGRF) on an ABI 3730L Q.S. with nuclease-free water. Amplification was performed using an Eppendorf MasterCycler epgradient S (Eppendorf, Hamburg, Germany) with the cycling conditions: 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. Products were sequenced by the Australian Genome Research Facility (AGRF) on an ABI 3730L using big dye terminator chemistry (Applied Biosystems, Foster City, CA, USA).

2.9. Data analysis

Gene expression levels were quantified with Cycle Threshold (Ct) values, which are the raw data from the real-time PCR, and refer to the number of PCR cycles required for the fluorescence signal to cross a threshold line. Ct values are inversely proportional to the amount of target nucleic acid, so the greater the Ct value, the lower the amount of target nucleic acid in the sample. All analyses were performed on mean Ct values, which were calculated from the three sample replicates used in the real-time PCR. Analysis of Variance (ANOVA) was used to test for differences in mean Ct values among treatments for each gene and tissue type combination. For these analyses, data from the two procedural replicates were pooled for each gene, and differences in expression were modest, ranging from 1.1- to 3.5-fold across treatments in both tissue types (Fig. 4A and 4B).

## 3. Results

Total amount of RNA extracted from brain tissue ranged from 202.5 to 673.2 ng, and heart tissue ranged from 217.8 to 990.0 ng, averaging 6.26 ng/mg and 5.06 ng/mg, respectively. All real-time PCR measurements for Hsp60, Hsp70, and Hsp90 were compared to the established reference gene 18 s (Hillis and Dixon, 1991), which did not change mRNA expression levels across heat shock treatments in heart (F_{4,24}=1.30, P=0.307; Fig. 2) or brain tissue (F_{4,24}=1.37, P=0.271; Fig. 3).

3.1. Hsp60 mRNA expression levels in heart and brain tissue

Levels of Hsp60 mRNA varied significantly among treatments in whole brain tissue (F_{4,24}=5.68, P=0.002), but not in heart tissue (F_{4,24}=0.74, P=0.578). For the whole brain tissue, pairwise t-tests revealed significant differences between treatment 4 (36 °C for 3 h, the highest heat shock temperature for the longest time) and all other treatments, including the control treatment. No other significant pairwise differences were detected in the whole brain tissue (Fig. 3). Relative to the control temperature, changes in Hsp60 mRNA expression were modest, ranging from 1.1- to 3.5-fold across treatments in both tissue types (Fig. 4A and 4B).

3.2. Hsp70 mRNA expression levels in heart and brain tissue

There were significant differences in the relative level of Hsp70 mRNA among treatments in both heart (F_{4,24}=2.92, P=0.048) and brain tissue (F_{4,24}=3.99, P=0.013). For heart tissue, pairwise comparisons revealed significant differences in mean Ct values between the control and treatment 4 (36 °C for 3 h) and between treatment 1 (34 °C for 1 h, the lowest heat shock temperature for the shortest time) and treatment 4 (Fig. 2). For brain tissue, pairwise comparisons revealed significant differences in mean Ct values between treatment 4 and all other treatments, including the control (Fig. 3). No other significant pairwise differences in mean Ct values were detected in either tissue. Relative to the control, mRNA expression of Hsp70 increased from 2.7- to 38.8-fold across treatments in heart tissue (Fig. 4A) and from 4.1 to 15.7-fold in the whole brain tissue (Fig. 4B). For both tissues, changes in Hsp70 expression were greatest in treatments 3 (34 °C, 3 h) and 4 (36 °C, 3 h).

3.3. Hsp90 mRNA expression levels in heart and brain tissue

As found for Hsp70, there were significant differences in levels of Hsp90 mRNA among treatments in both heart (F_{4,24}=16.89, P=0.001) and brain tissue (F_{4,24}=24.68, P<0.001). For heart tissue, pairwise comparisons revealed significant differences between the control and all heat shock treatments, as well as between treatments 1 and 4 and between treatments 3 and 4 (Fig. 2). For brain tissue, there were significant differences between the control and all heat shock treatments, as well as between treatments 1 and 3, between treatments 1 and 4, between treatments 2 and 4 and between treatments 3 and 4 (Fig. 3).

Relative to the control, mRNA expression of Hsp90 increased 14.8- to 98.3-fold across treatments, with the greatest increase occurring in treatment 4 (36 °C, 3 h) (Fig. 4A). For whole brain
tissue differences in expression ranged from 3.3 to 14.7-fold across treatments. Again, the greatest increase in expression was in treatment 4 (Fig. 4B).

3.4. Hatching success of heat shocked embryos relative to controls

Embryos from all treatments hatched after 53–57 days of incubation. The average time to hatching of embryos that experienced a heat shock on Day 25 was 56 days \((n=21)\), while the average time to hatching of embryos that experienced a heat shock on Day 45 was 55 days \((n=33)\). Hatching success of ‘Day 25 shock’ embryos averaged 65.6% across all treatments (procedural controls and heat shock treatments), while the hatching success of ‘Day 45 shock’ embryos was 72.9% when averaged across treatments (Table 2). There was a marginally significant difference in hatching success among treatments in the Day 25 embryos exposed to a one-hour heat shock. However, there were no significant differences among treatments in the Day 25 embryos exposed to a three-hour heat shock or the Day 45 embryos (Table 2).

4. Discussion

Our study provides the first evidence that *C. caretta* embryos subjected to heat shocks upregulate their transcription of genes that encode for heat-shock proteins known to ameliorate some of the negative effects of temperature on metabolic processes. As all treatment groups successfully hatched, the heat shock treatments were non-lethal and effectively induced a thermotolerance response in the embryos. Expression differed in magnitude between heart and brain tissues, across treatments and among genes. The most severe treatment \(T4; 36\,\textdegree\text C\) shock for 3 h\) provided the most effective set of conditions for inducing an increase in gene expression of *Hsp70* and *Hsp90*. In contrast, fold-changes were much more subdued in *Hsp60* in both tissue types, suggesting that this gene is not a good indicator of a physiological response to acute heat stress. Although we did not quantify heat-shock protein production in our study, previous studies have shown that protein expression and gene expression levels for *Hsp60*, *Hsp70*, and *Hsp90* are correlated (de Sousa Abreu et al., 2009; Ghazalpour et al., 2011), albeit with a caveat that mRNA abundances are not direct reflections of protein abundances as most protein expression is regulated during translation or degradation (de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012). We therefore conclude that *Hsp70* and *Hsp90* mRNA are effective biomarkers for measuring a physiological response to heat stress in *C. caretta*.

4.1. Expression of *Hsp60* in heart and brain

We found no significant changes in relative expression of *Hsp60* in embryonic heart tissue in response to temperature treatments. This result implies that *Hsp60* expression at the genetic level is stable during development. *Hsp60* and other larger kDa proteins in the heat shock family are highly conserved across all taxa (Iwama et al., 1998), but their expression profiles can vary by tissue type and treatment design. Marber et al. (1995) demonstrated that expression of *Hsp60* does not significantly alter in mammal hearts under ischaemic conditions where blood flow was restricted. Later work on heart tissue of adult fresh water turtles (Chang et al., 2000; Kesaraju et al., 2009) and canines (Chang et al., 2000) in low oxygen environments also found no difference in the expression of *Hsp60* between stress and basal levels. In contrast, Van et al. (2009)

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**Fig. 2.** Comparison of 18s, *Hsp60*, *Hsp70*, and *Hsp90* mean Ct values \((\pm\, S.E.)\) in whole heart tissue of Day 45 embryos for each treatment \((n=3-5)\). Statistically significant differences \((P<0.05)\) among treatment groups are indicated with dissimilar letters.
Fig. 3. Comparison of 18s, Hsp60, Hsp70, Hsp90 mean Ct values (± S.E.) in whole brain tissue of Day 45 embryos for each treatment (n=3-6). Statistically significant differences (P<0.05) among treatment groups are indicated with dissimilar letters.

Fig. 4. Changes in levels of Hsp60, Hsp70, Hsp90 mRNA in whole heart tissue (A) and whole brain tissue (B) of Day 45 embryos after heat shock Treatment 1 (34°C, 1 h), 2 (36°C, 1 h), 3 (34°C, 3 h), and 4 (36°C, 3 h) as outlined in Fig. 1 (heart n=24, brain n=29). Values are presented as mean fold-change (± S.E.) relative to procedural control embryos.
found Hsp60 protein expression in the hearts of one-day old chicks (Gallus gallus) gradually increased after a 5-hour heat shock of 37 °C. Unlike the heart tissue, we found modest yet significant changes in relative Hsp60 expression across treatments in whole brain tissue. This implies that Hsp60 expression in brain tissue has a higher temperature-sensitivity than heart tissue. Considering changes in relative expression in both tissue types, our experimental conditions produced a low to modest response to heat stress, but were significant only in brain tissue. Therefore, we conclude that Hsp60 is not well suited to act as a biomarker for thermotolerance responses to acute heat stress, but may be an effective biomarker with chronic heat stress conditions.

### 4.2. Expression of Hsp70 in heart and brain

We found that the expression of Hsp70 in embryonic C. caretta hearts was temperature-dependent and greatest at the highest temperature treatment in both tissue types. Li et al. (2012) also observed that relative expression of Hsp70 increased in heart tissue of adult Chinese soft-shelled turtles (Pelodiscus sinensis) subjected to a heat shock at 40 °C for 4 hours. Not only is Hsp70 responsive to heat stress, but there is also evidence of cold sensitivity. Stecyk et al. (2012) show an increase of expression in cardiac tissue of adult freshwater turtles (Trachemys scripta) acclimated to cold (5 °C) anoxic environments. However, there were no significant changes in relative expression in telencephalon with cold acclimation (Stecyk et al., 2012). This could indicate that Hsp70 is an important protein for maintaining cardiac function under stressful conditions.

Although we found that heat shock treatments induced different levels of upregulation of Hsp70 in different tissues, increased expression relative to controls was a consistent response across all treatments and tissue types. Our observation is consistent with that of Feder and Hofmann (1999) that changes in Hsp70 expression can be used as a biological indicator of a response to thermal stress in embryonic sea turtles.

### 4.3. Expression of Hsp90 in heart and brain

Hsp90 is a chaperone protein comprising of 1–2% of all soluble proteins in the cytoplasm (Benjamin and MacMillan, 1998). Its expression is very sensitive in reptiles and can vary in both a temperature-and time-dependent manner in response to environmental stressors (Kohn et al., 2010; Ramaglia and Buck, 2004; Stecyk et al., 2012). We found significant increases in expression of Hsp90 in both heart and brain tissue, suggesting that low basal levels of the gene are active in these tissues and readily amplified in response to acute heat stress. In both tissues, expression of Hsp90 was temperature-dependent, but in brain tissue, activity was also dependent on the duration of the heat shock. Hsp90 protein is abundant in turtle brain tissue (Ramaglia and Buck, 2004), as it is necessary for protection against neuronal death (Mailhos et al., 1994; Pratt, 1998; Wyatt et al., 1996). Our findings suggest that regardless of its abundance in various tissues, Hsp90 is extremely sensitive to environmental stressors during embryonic development. Therefore, it readily changes expression in response to heat stress experienced in the nest and is a relevant biomarker for determining a thermotolerance response for developing sea turtles.

### 4.4. Post-heat shock hatching success

Using a non-linear relationship between temperature and embryonic development rate fitted to data for Western Australian C. caretta (Woolgar et al., 2013), we calculated that Day 25 and Day 45 embryos had completed approximately 53 ± 2% and 86 ± 2% of their development to hatching at the time of their respective heat shocks. Hence, we exposed embryos to stressful temperatures near the sub-lethal limit of 35 °C, and within a time frame where they were potentially most vulnerable during later stages of development (Davenport, 1997).

Although there was high variation, there were no significant differences detected between the hatching success of heat shocked and control treatments for all embryos, confirming our incubation design effectively produced a stress response without causing mortality. A marginally significant effect was found in Day 25 embryos exposed to a one-hour heat shock, but the effect was no longer significant after controlling for multiple comparisons with Bonferroni corrections. It is also noteworthy that the hatching success in Day 25 embryos exposed to a one-hour heat shock did not vary between the control and the most extreme heat shock temperature and there was no significant effect in Day 25 embryos exposed to a three-hour heat shock. These results support our expectation that exposure to 34 °C is sub-lethal (Davenport, 1997).

Similarly, we have evidence that brief exposure to 36 °C is also sub-lethal, though we did note that hatchlings in this treatment emerged lethargic and hunched in posture. As the natural survival rate for sea turtle embryos is around 80% (Bolten et al., 2011; Miller, 1985; Ozdemir et al., 2008), a hatching success of 75% for the most extreme temperature and duration of the heat shock indicates that this treatment produced a thermotolerance response in late-stage embryos without causing mortality.

Post-hoc analysis of the sex ratios of 119 randomly selected hatchlings from this study resulted in a 1:1 ratio of males to females (Woolgar et al., 2013). This was expected as embryos were incubated at their pivotal temperature of 29.0 °C until (1) point of sacrifice or (2) the heat shock was applied. The sex determination period for this population of C. caretta is between 33-64% of development to hatching stage (Woolgar et al., 2013). As our heat shocks were given either during (Day 25, 53% developed) or after (Day 45, 86% developed) the point of gonadal differentiation, it is possible that the sex of the hatchling could have influenced gene expression. However, as the individuals for which we had gene expression data were not sexed, we were unable to test this.

### 4.5. Conclusions

Given the challenges that ecosystems face as a result of rapid environmental change, methods to assess the effects of these processes at the individual to ecosystem levels are urgently required. At the individual level, gene expression analyses are powerful tools for assessing the response of an organism to changing environments. By simulating acute heat stress in C. caretta embryos, we successfully elicited a heat stress response above a control group. Relative expression of Hsp70 and Hsp90 significantly increased in both heart and brain tissue in reaction to heat stress, and we conclude that these genes are appropriate biomarkers for measuring a thermotolerance response in C.
in embryos and potentially other species of sea turtle. The extent of this resilience requires further research to investigate how proteins are upregulated under higher or longer periods of heat stress, and whether hatching success is compromised under more extreme conditions. The capacity for reptile embryos to acclimatize to warmer nesting environments through changes in their phenotype is an important area for further study given the current pace of environmental change.

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References


Reconstructed paternal genotypes reveal variable rates of multiple paternity at three rookeries of loggerhead sea turtles (Caretta caretta) in Western Australia

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Abstract. Female sea turtles are promiscuous, with clutches of eggs often sired by multiple males and rates of multiple paternity varying greatly within and across species. We investigated levels of multiple paternity in loggerhead sea turtles (Caretta caretta) from three rookeries in Western Australia by analysing polymorphic species-specific genetic markers. We predicted that the level of multiple paternity would be related to female population size and hence the large rookery at Dirk Hartog Island would have higher rates of multiple paternity than two smaller mainland rookeries at Gnaraloo Bay and Bungelup Beach. Contrary to our prediction, we found highly variable rates of multiple paternity among the rookeries that we sampled, which was unrelated to female population size (25\% at Bungelup Beach, 86\% at Gnaraloo Bay, and 36\% at Dirk Hartog Island). Approximately 45 different males sired 25 clutches and the average number of sires per clutch ranged from 1.2 to 2.1, depending on the rookery sampled. The variance in rates of multiple paternity among rookeries suggests that operational sex ratios are variable in Western Australia. Periodic monitoring would show whether the observed patterns of multiple paternity for these three rookeries are stable over time, and our data provide a baseline for detecting shifts in operational sex ratios.

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Introduction

Unlike many bird and mammal species, parental care beyond nesting is absent in most reptiles (Shine 2005; Uller and Olsson 2008). Males do not provide any resources to females other than sperm, yet multiple paternity in clutches has been recorded in most reptile species to date (Uller and Olsson 2008). Multiple paternity has been detected in all seven extant species of sea turtle, with one or two sires being the most common number for a single clutch (reviewed by Bowen and Karl 2007, and Lee 2008). In sea turtles, multiple paternity can arise in two ways: either a female can mate with more than one male during the same reproductive cycle or, alternatively, a female may utilise sperm stored from a previous breeding season (Pearse and Avise 2001; Lara-De La Cruz et al. 2010; Phillips et al. 2014a).

Many explanations for multiple paternity have been proposed, including increased fertilisation success, improved offspring fitness, and harassment of receptive females by males (Jensen et al. 2013). Ireland et al. (2003) and Lee and Hays (2004) suggested that the phenomenon was a product of male density and avoidance of aggressive mating behaviour by females, causing females to mate with more than one male (convenience polyandry). A study on solitary and mass-nesting (arribada) Olive Ridley turtles (Lepidochelys olivacea) by Jensen et al. (2006) attributed the higher rate of multiple paternity in the arribada females to their high density of nesting. As males rarely come ashore and are difficult to catch at sea, genetic analyses of nesting females and their offspring can both identify the number of sires per clutch and provide data on the number of breeding males and females from which operational sex ratios (OSRs) can then be calculated (Wright et al. 2012a, 2012b; Hawkes et al. 2014). The OSR of a given population should be proportional to the number of males at the breeding
area before the nesting season (Hays et al. 2010; Stewart and Dutton 2011), and therefore reflect the underlying genetic variation of the population.

Relative to other parts of the world, little is known about the population dynamics of loggerhead turtles (Caretta caretta) nesting in the eastern Indian Ocean. In Australia, there are two genetically distinct populations of C. caretta, one in Western Australia and the other in Queensland (Baldwin et al. 2003). All rookeries in Western Australia comprise a single genetic stock (Pacioni et al. 2012), spanning ~520 km of coastline from Dirk Hartog Island (25.49827°S, 112.98719°E) at the southern limit to the Muiron Islands north-east of Exmouth (21.39156°S, 114.21205°E) at the northern limit of the range (Baldwin et al. 2005). Although the rookeries within this area constitute the third-largest population of C. caretta in the world (Baldwin et al. 2003; Reinhold and Whiting 2014), we know relatively little about the population demographics. A description of mating systems, quantification of the incidence of multiple paternity, and quantification of genetic variation is a first step towards understanding the implications of climate change and changing sex ratios of this globally important population.

Genetic analyses offer a means to indirectly sample the male component of a population of breeding turtles (Lee 2008; Phillips et al. 2014b; Stewart and Dutton 2011) and there are several methods for estimating multiple paternity using genetic data (Table 1). For sea turtles, such studies show that rates of multiple paternity are highly variable (Table 1), though it is unclear whether the reported variability among species and

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**Table 1. Variation in rates of multiple paternity in sea turtles within species and across studies**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of clutches analysed</th>
<th>Mean no. offspring genotyped per clutch</th>
<th>No. loci analysed</th>
<th>Minimum no. of males</th>
<th>Frequency of MP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Methods</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green turtle (C. mydas)</td>
<td>Ascension Island</td>
<td>18</td>
<td>3.9</td>
<td>2–5</td>
<td>2</td>
<td>61% DADSHARE, GERUD</td>
<td>Lee and Hays (2004)</td>
</tr>
<tr>
<td></td>
<td>Ascension Island</td>
<td>3</td>
<td>1.5</td>
<td>2</td>
<td>2</td>
<td>100% REAP</td>
<td>Ireland et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Southern Great Barrier Reef</td>
<td>22</td>
<td>41.3</td>
<td>5</td>
<td>1</td>
<td>9% GENEPOP 3.1</td>
<td>Fitzsimmons (1998)</td>
</tr>
<tr>
<td></td>
<td>Tortuguero, Costa Rica</td>
<td>8</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>63% Irwin</td>
<td>Peare and Parker (1996)</td>
</tr>
<tr>
<td></td>
<td>Algadi, Cyprus</td>
<td>20</td>
<td>21.9</td>
<td>14</td>
<td>1.4</td>
<td>36% COLONY 2.0</td>
<td>Wright et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Sri Lanka</td>
<td>24</td>
<td>10</td>
<td>6</td>
<td>1.7</td>
<td>63% GERUD 2.0</td>
<td>Ekanayake et al. (2013)</td>
</tr>
<tr>
<td>Loggerhead turtle (C. caretta)</td>
<td>Zakynthos, Greece</td>
<td>15</td>
<td>40.7</td>
<td>4</td>
<td>3.2</td>
<td>93% GERUD 1.0</td>
<td>Zbinden et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Melbourne Beach, Florida</td>
<td>70</td>
<td>10</td>
<td>4</td>
<td>1.4</td>
<td>31% PARENTAGE</td>
<td>Moore and Ball (2002)</td>
</tr>
<tr>
<td></td>
<td>Mon Repos, Queensland</td>
<td>24</td>
<td>21</td>
<td>0</td>
<td>–</td>
<td>33% Allozymes</td>
<td>Hury and Briscoe (1988)</td>
</tr>
<tr>
<td></td>
<td>Melbourne Beach, Florida</td>
<td>3</td>
<td>20.7</td>
<td>2</td>
<td>–</td>
<td>33% –</td>
<td>Bollmer et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Nagoya, Japan</td>
<td>7</td>
<td>29</td>
<td>2</td>
<td>–</td>
<td>43% –</td>
<td>Sakanaka et al. (2011)</td>
</tr>
<tr>
<td>Olive Ridley turtle (L. olivacea)</td>
<td>Ostional, Costa Rica</td>
<td>13</td>
<td>22</td>
<td>2</td>
<td>2.8</td>
<td>92% GERUD 1.0</td>
<td>Jensen et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Playa Hermosa, Costa Rica</td>
<td>13</td>
<td>22.6</td>
<td>2</td>
<td>1.4</td>
<td>30% GERUD 1.0</td>
<td>Jensen et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Galibi, Suriname</td>
<td>10</td>
<td>70.3</td>
<td>2</td>
<td>1.2</td>
<td>20% Initial inference</td>
<td>Hooe et al. (2002)</td>
</tr>
<tr>
<td>Kemp’s Ridley turtle (L. kempii)</td>
<td>Tamaulipas, Mexico</td>
<td>26</td>
<td>7.8</td>
<td>3</td>
<td>–</td>
<td>58% –</td>
<td>Kishler et al. (1999)</td>
</tr>
<tr>
<td>Hawkbill turtle (E. imbricato)</td>
<td>Goliassam, Sabah, Malaysia</td>
<td>10</td>
<td>27</td>
<td>3</td>
<td>1.3</td>
<td>20% GERUD 1.0</td>
<td>Joseph and Shaw (2011)</td>
</tr>
<tr>
<td></td>
<td>Cousine Island, Seychelles</td>
<td>43</td>
<td>18.8</td>
<td>33</td>
<td>–</td>
<td>9.3% COLONY 2.0</td>
<td>Phillips et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Seychelles (various islands)</td>
<td>249</td>
<td>22.6</td>
<td>32</td>
<td>–</td>
<td>9.2% COLONY 2.0</td>
<td>Phillips et al. (2014a)</td>
</tr>
<tr>
<td>Leatherback turtle (D. coriacea)</td>
<td>Las Baulas, Costa Rica</td>
<td>4</td>
<td>–</td>
<td>2</td>
<td>1</td>
<td>0% –</td>
<td>Rieder et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Sandy Point, Virgin Islands</td>
<td>38</td>
<td>26.8</td>
<td>7</td>
<td>–</td>
<td>42% GERUD 1.0</td>
<td>Stewart and Dutton (2011)</td>
</tr>
<tr>
<td>Flatback turtle (N. depressus)</td>
<td>Mon Repos and Peak Island, Queensland</td>
<td>16</td>
<td>26.7</td>
<td>4</td>
<td>–</td>
<td>69% Initial inference, COLONY 2.0</td>
<td>Theissinger et al. (2009)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Arribada nesting beach.
<sup>b</sup>Solitary nesting beach.
<sup>c</sup>Captive population, paternal genotype known.
populations is due to the use of different types of genetic markers, differences in multiple paternity estimation methods, or if indeed it reflects natural variability among populations. To date, only one study (Jensen et al. 2006) has concurrently examined the frequency of multiple paternity in two different rookeries of the same species.

Here, we investigated patterns of multiple paternity in clutches sampled from three locations spread across the geographic range of rookeries of *C. caretta* in Western Australia. The southern-most rookery was on Dirk Hartog Island (DHI), one of the world’s largest rookeries (Baldwin et al. 2003; Reinhold and Whiting 2014), while we also sampled clutches from near the northern-most edge (Bungelup Beach, BB) and from a smaller mainland rookery approximately midway in the breeding range (Gnaraloo Bay, GB). We aimed to describe: (1) the presence of multiple paternity, and (2) spatial variation in multiple paternity rates among rookeries across the range of the nesting population. Because these rookeries differed in size, we predicted that the frequency of multiple paternity should be higher in clutches from the larger nesting rookery at DHI compared with the smaller, mainland rookeries (GB and BB) based on the density-dependence convenience polyandry model. To exclude the possibility that any variation in multiple paternity we detected was an artefact of methodology, we analysed paternity using identical statistical methods and the same genetic markers for samples from all rookeries. Our results are discussed in the context of estimating population size and the implications of climate change on the demography of the Western Australian population of *C. caretta*.

**Methods**

**Egg collection and tissue sampling**

Eggs of *C. caretta* were collected from three rookeries in Western Australia during peak nesting periods between 2011 and 2013. Collection sites and dates were Turtle Bay on Dirk Hartog Island (25.49827°S, 112.98719°E) in January 2013, Gnaraloo Bay on the Western Australian mainland (23.82618°S, 113.52629°E) in January 2011 (Woolgar et al. 2013), and Bungelup Beach in the Cape Range National Park on the Exmouth Peninsula (22.282331°S, 113.831570°E) in December 2013 (Fig. 1). The Dirk Hartog Island rookery hosts the largest nesting numbers,
with ~2000 nesting females per season (Trocini 2013; Reinhold and Whiting 2014), while an estimated 700–1200 females nest per season at Bungulup Beach (Trocini 2013). In contrast, nesting at the Gnaraloo Bay rookery is comparatively infrequent, with ~100 females per season (Hattingh et al. 2011).

All offspring samples in this study were used opportunistically, and they were collected for other research projects (Woollgar et al. 2013; Tedeschi et al., unpubl. data). As a consequence, sample sizes varied among clutches and among rookeries. We had access to 80 eggs per clutch (n = 15 clutches) from the Dirk Hartog Island rookery, and 20 eggs per clutch (n = 4 clutches) from Bungulup Beach. Eggs sampled from these rookeries were incubated in the laboratory and embryos were euthanised before hatching. Maternal tissue was collected from these two rookeries during oviposition by sampling from the trailing edge of the back flipper with a sterile 3-mm biopsy punch (Byland Medical, NSW, Australia). At the Gnaraloo Bay rookery, hatchlings were collected from nests for a study conducted by Woollgar et al. (2013), and we used available samples to assess paternity (GB; n = 10–22 eggs per clutch from eight clutches). No maternal tissue samples were available for the GB rookery because the collection permit for the study on this population did not cover sampling of adult females. All samples from the DHI (n = 859) and BB (n = 66) rookeries were stored at room temperature in 2 mL Longmire buffer until processing, whereas samples from the GB rookery (n = 119) were stored at 4°C in 1.5-2.0 mL of 100% ETOH.

Microsatellite analysis and genotyping

Fourteen of the 15 clutches collected from DHI were genotyped, as one clutch was unfertilised. Total DNA was extracted from 1026 samples of offspring (minimum of 10 offspring per clutch) and 18 maternal samples using a standard salting-out method (Sunnucks and Hales 1996), with the exception of proteinase K digestion [200 μg mL \(^{-1}\)] at 56°C overnight. The DNA pellet was resuspended in 100 μL nuclease-free sterile water and quantified by a NanoDrop® Spectrophotometer (ND1000, Thermo Fisher Scientific, Australia). All samples were normalised to 10 ng DNA μL \(^{-1}\) with nuclease-free water before polymerase chain reaction (PCR).

Four loci designed for M. caretta (CcS697, Cc7B07, Cc5F01, Cc7C04; see Shamblin et al. 2007) were run in a single PCR multiplex. PCR was performed in 10-μL reactions with 1 ng DNA template, 7.8 μL Platinum Supermix (Invitrogen, Life Technologies, Vic., Australia), 0.2 μL MgCl₂ [50 mM], and 0.25 μL each primer [7 μM]. PCR products were denatured at 95°C for 3 min, (40 × 30 s at 95°C, 45 s at 53°C, 30 s at 72°C, and 8 min extension at 72°C. All PCR products were analysed on an ABI 3730 Sequencer against GeneScan 500 LIZ internal size standard and DNA fragments were scored manually with GeneMarker 1.91 software (SoftGenetics, LLC®, USA).

Data analysis

Levels of genetic variation among the 18 maternal genotypes (DHI and BB rookeries) were assessed by calculating the number of alleles per locus, and allele frequencies at each locus using the GENEALEX 6.5 software package (Peakall and Smouse 2012). We also used this program to assess Hardy–Weinberg equilibrium and calculate the probability of two different females having identical multilocus genotypes. Observed and expected heterozygosity for the four loci for each rookery were estimated with CERVUS 3.0.3 (Marshall et al. 1998). The presence of null alleles was tested at each locus in only the 14 maternal genotypes from the DHI rookery using the software package MICROCHECKER (Vane Oosterhout et al. 2004); the sample size from the BB rookery was insufficient for detecting null alleles with reliability.

We assessed paternity within each clutch sample using initial inference, the GERUD 2.0 software package (Jones 2005), and the COLONY 2.0 software package (Wang 2004; Wang and Santure 2009). Neither GERUD 2.0 nor COLONY 2.0 require population allele frequencies in order to calculate the minimum number of sires (Jones 2005; Wang and Santure 2009), so these packages were ideal for our purposes given that other adult females from the rookeries were not sampled.

To evaluate paternity with initial inference we used the maternal genotypes to identify maternal contributions to each offspring and inferred paternal alleles by excluding maternal alleles in the offspring genotypes (Jones et al. 2010). Multipaternity was determined when three or more non-maternal alleles were found at a single locus. Since maternal genotypes were not available for the GB rookery, we inferred maternal allelic contribution based on the frequency of alleles in the offspring within each clutch. The GERUD 2.0 analyses were performed using all four loci with the parameter for the maximum number of sires set to four. Runs were conducted with and without maternal genotypes. When the GERUD program returned multiple solutions for progeny arrays, they were ranked by likelihood based on the segregation of paternal alleles and their deviation from Mendelian expectations (Jones 2005). The combination of sires with the highest probability score was used to calculate the minimum number of sires for the clutch.

The COLONY analyses were also performed using all four loci. COLONY assigns sibships and parentage based on a maximum-likelihood model. Offspring are clustered by full-sib and half-sib (maternal and paternal), and parent-offspring relationships are determined, with parents assigned to full-sib groups. Unknown genotypes for either parent can be inferred (Wang 2004; Wang and Santure 2009). For each rookery, all genotyped offspring were analysed in a single dataset to identify any paternal half-sibs, which would indicate males that sired offspring with more than one female. COLONY was set to the default parameters, a single medium-length run, with full-likelihood analysis, assuming polygamy for both males and females. Parallel to the GERUD analysis, COLONY runs were performed with and without maternal genotypes. COLONY can estimate paternity with datasets containing missing or rare alleles, but GERUD cannot. Offspring for which maternal alleles or data were missing were therefore excluded from the GERUD analysis. The reduced dataset for the DHI rookery included genotypes for 14 females and 791 offspring; full dataset included 813 offspring. For BB, the reduced dataset was for 4 females and 69 offspring; full dataset included 62 offspring. Finally, for the GB rookery, the reduced dataset included 84 offspring while the full dataset analysed included 92 offspring. Following the consensus approach proposed by Theissinger et al. (2009) and Stewart and Dutton (2011), multiple
paternity was identified in each clutch if two of the three methods used had detected more than one sire.

**Results**

All four loci were polymorphic, with the number of alleles per locus ranging from 6 to 22, with observed heterozygosity ranging from 0.70 to 1.00 (Table 2). The probability of females from the BB and DHI rookeries sharing a multilocus genotype ranged from 1.6 × 10⁻² to 7.5 × 10⁻². Genotypic frequencies for the DHI rookery at all loci were in agreement with Hardy–Weinberg equilibrium \( P > 0.05 \), and no null alleles were detected.

The estimated proportions of multiple paternity varied among rookeries (Table 3). On the basis of initial inference, the frequency of multiple paternity was 25.0% (1 of 4 clutches) at BB, 35.7% (5 of 14 clutches) at DHI and 85.7% (6 of 7 clutches) at GB. The mean minimum number of sires per clutch estimated using initial inference ranged from 1.2 to 1.9 (Table 3).

Estimates of multiple paternity and the minimum number of sires per clutch were slightly higher when calculations were based on the GERUD and COLONY analyses. The frequency of multiple paternity ranged from 25% (1 of 4) to 100% (7 of 7) and the minimum number of sires per clutch ranged from 1.1 to 2.1 (Table 3). Nevertheless, a similar pattern to the initial inference estimates was apparent, with both estimates for the large rookery at DHI being closer to the lower range values. The GERUD estimates of minimum number of sires per clutch and frequency of multiple paternity were identical when calculated with or without maternal genotypes. Two additional instances of multiple paternity were detected in the COLONY analyses when runs were conducted without maternal genotype (Table 3).

Reconstructed paternals from GERUD and COLONY agreed six out of the 12 instances (50%) where multiple paternity was determined across the three rookeries. The analyses indicated that 16–25 individual males sired offspring in the clutches sampled from the DHI rookery (\( n = 14 \)), 5 males sired offspring in the clutches from BB (\( n = 4 \)), and 11–15 males sired offspring in the clutches from the GB rookery (\( n = 7 \)). None of the males were identical across the three rookeries. Where maternal genotypes were known, the probability of two males from the BB and DHI rookeries sharing a multilocus genotype ranged from 5.5 × 10⁻³ to 9.2 × 10⁻². Where the maternal genotype was not known, the probability of two males from all three rookeries sharing a multilocus genotype ranged from 1.6 × 10⁻² to 9.2 × 10⁻².

**Discussion**

At three *C. caretta* rookeries in Western Australia, females laid clutches that were sired by multiple males 25–86% of the time during peak nesting periods between 2011 and 2013. This result is consistent with estimates of multiple paternity in populations of *C. caretta* from the Northern Hemisphere and eastern Australia, where rates of 25–33% are typical (Table 1).

The highest rate of multiple paternity was found at Gnaraloo Bay (GB) where multiple males sired 86% of clutches (assuming paternal genotypes were correctly deduced from correctly inferred maternal genotypes). It is unclear why such a high rate of multiple paternity should occur in a low-nesting rookery such as GB, although the size of the offshore breeding area occupied by males and females may impact the nesting density, and hence male–female encounters. For example, Zhinden et al. (2007) reported that 93% of *C. caretta* clutches from the Laganas Bay rookery on Zakynthos Island in Greece exhibited multiple paternity. They attributed this rate to the small size of the bay that bordered the nesting beach, which confined the population and increased densities of breeding males and females. Lasala et al. (2013) also report a high rate of multiple paternity (75%) for nests on Wassaw Island, Georgia. The authors suggest that this may be due to a large number of males migrating along the coastline and crossing nesting beach boundaries. The GB rookery is situated on a wide and open bay with near-continuous fringing reef (Short 2005; Hattingh et al. 2011), so it is plausible that the high rate of multiple paternity found at this rookery is a result of large numbers of males migrating along the fringing reefs. However, as we do not know how much of the offshore area comprises the breeding grounds, tracking of sea turtles in the water during the breeding season would indicate the density of turtles at sea, and permit estimation of the probability of male–female encounters (Schofield et al. 2013).

In contrast to the rookery at GB, the frequency of multiple paternity was lower in clutches sampled from the BB (25%) and DHI (36%) rookeries. Although the estimate of low multiple paternity for the BB rookery may reflect the relatively small clutch and offspring sample sizes, this was not the case for DHI, where sample sizes and the number of clutches analysed were comparatively large (see Table 1). Uller and Olsson (2008) proposed that, all else being equal, the degree of multiple paternity should be positively correlated with the probability of male encounters. If this model applies to *C. caretta* in Western Australia, it would imply that male density is higher in the centre of the species’ Western Australian distribution. However, additional survey and molecular work would be required to verify this possibility.
We found no evidence of a relationship between rookery size and the incidence of multiple paternity as has been reported elsewhere in sea turtles (Lee 2008). The *C. caretta* rookery at DHI is one of the largest in the world (Baldwin et al. 2003; Reinhold and Whiting 2014), but has one of the lowest rates of multiple paternity reported. Phillips et al. (2013, 2014b) found a similar pattern in a population of hawksbill turtles (*Eretmochelys imbricata*) nesting in the Seychelles Islands. They found a high number of males contributing to the clutches sampled (47 males fertilised 43 clutches), but the frequency of multiple paternity was low (9.3%), which they attributed to a low rate of mate encounter over a widely dispersed breeding area (Phillips et al. 2013, 2014b). The lack of a relationship between rookery size and rates of multiple paternity might also reflect a declining number of males associated with feminisation of primary sex ratios due to climate change (Wright et al. 2012; Hawkes et al. 2014). However, no data on the trends in male abundance in this genetic stock are available to support this assumption.

Table 3. Minimum number of sires per clutch in *C. caretta* as estimated by initial inference, GERUD 2.0, and COLONY 2.0 runs with and without maternal genotype

<table>
<thead>
<tr>
<th>Rookery</th>
<th>Clutch</th>
<th>No. of embryos analysed (clutch size)</th>
<th>Initial inference</th>
<th>GERUD 2.0</th>
<th>COLONY 2.0</th>
<th>Multiple paternity?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>With maternal genotype</td>
<td>Without maternal genotype</td>
<td>With maternal genotype</td>
</tr>
<tr>
<td>DHI</td>
<td>A</td>
<td>41 (45)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>60 (69)</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>37 (42)</td>
<td>1</td>
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<td></td>
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<td>1.26 ± 0.13</td>
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<tr>
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<td></td>
<td>1.25 ± 0.25</td>
<td>25% (1/4)</td>
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<tr>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>71.4% (5/7)</td>
<td>100% (7/7)</td>
<td>42.9% (3/7)</td>
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Hence, as *C. caretta* has temperature-dependent sex determination, with males being produced at cooler incubation temperatures (Miller 1985; Standora and Spotila 1985; Menovsky 1994), the GB rookery, at the centre of the species’ range, may produce relatively more male offspring than at the other two rookeries studied (see Woolgar et al. 2013). This, in turn, could drive differences in the OSRs of each nesting population. Further, male-mediated gene flow is promoted by mating on migration routes and possibly feeding grounds (Fitzsimmons et al. 1997b), which may contribute to sex ratio differences between rookeries, especially if females and males travel different routes (Fitzsimmons et al. 1997b; Wright et al. 2012b). Measuring nest incubation temperatures across years to assess long-term changes in hatching sex ratios (Laloé et al. 2014) in combination with long-term genetic monitoring of the nesting females will show whether the pattern we observed for these three rookeries is temporarily stable, and our data can be used as a baseline for determining whether OSRs change over time.

Two common methods for estimating OSRs in sea turtle populations are to count the number of females and males encountered along a transect (Hays et al. 2010) or to estimate paternal contributions of clutches sampled from nesting beaches (Stewart and Dutton 2011; Hawkess et al. 2014). OSRs change as the nesting season progresses, as males and females arrive at breeding grounds at different times, have different periods of residence, and different remigration intervals (Limpus 1993; Godley et al. 2002; Hays et al. 2010, 2014). As the remigration interval for male *C. caretta* is shorter than for females, future scenarios of climate change may not decrease population viability even with increased feminisation of offspring (Hays et al. 2010; Phillips et al. 2014b; but see Wright et al. 2012a). As long as males return frequently to breeding grounds, fertilisation success should be stable (Hays et al. 2010; Wright et al. 2012a). Hence, healthy and genetically diverse populations should be able to absorb a reduction in males given the polyandrous nature of sea turtles, but periodic monitoring of OSRs (e.g. every 5–10 years) is critical for detecting ratios that could reduce population viability.

In summary, it is clear that multiple paternity is the normal mating system in most species of sea turtle, and that rates vary by species, population, and by the method of detection (Bowen and Karl 2007). Despite the lack of a maternal genotype for one of our three rookeries, all methods used in this study led us to conclude that multiple paternity rates ranged from 25 to 86% in *C. caretta* clutches sampled from Western Australian rookeries. Additional samples from BB and GB (including maternal tissue), as well as from the Muiron Islands at the northern limit of the nesting range, would be valuable for assessing whether our estimates of the rates of multiple paternity are representative of the Western Australian population. Further, reconstruction of the genotypes of males that successfully mated with females, as we did in this study, allows the indirect estimation of the number of males contributing to this population and hence more realistic estimation of the adult population size. The rates of multiple paternity we have detected provide a snapshot of the mating system of the Western Australian population, and it will be important to repeat our sampling in order to detect changes in OSRs over time.

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**References**


