Corticosterone secretion in tuatara (*Sphenodon punctatus*): Influential factors and conservation applications

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ABSTRACT

Animals are regularly exposed to environmental, social and physiological challenges. In reaction to these challenges, individuals adjust their physiology and behaviour to maintain essential processes and optimise fitness. The most widely used indicators of physiological stress in vertebrates are glucocorticoid hormones (corticosterone (CORT) or cortisol), which are commonly referred to as 'stress hormones'. The use of CORT as a tool to understand how individuals respond to natural or human-caused challenges is central to stress physiology research. Here, I investigated intrinsic and extrinsic factors associated with CORT secretion, CORT secretion as an indicator of physiological response to challenges/stressors, and the value of CORT secretion as conservation tool in an iconic protected reptile (the tuatara, *Sphenodon punctatus*).

A capture-restraint time series revealed a significant CORT response over a 24 h period in male and female (non-gravid and gravid) tuatara. Baseline CORT and the CORT response to capture and restraint (i.e. a standardised capture-stress protocol) were similar between sexes; however, female reproductive condition was correlated with CORT secretion in that higher baseline CORT and a lower CORT response were observed in gravid females. An observational study incorporating data across a wide range of ambient temperatures (from four island sites) confirmed that body temperature (T_b) is positively correlated with baseline CORT in gravid females only, and revealed a positive correlation between the CORT response and higher T_b in all adults. A supporting experimental study showed that acute ambient temperature increase (in which mean T_b reached 21.4±0.4°C) elicits a significant CORT response to capture-restraint in gravid females. These results confirmed that gravid females are not secreting CORT maximally during nesting, but actively modulate secretion. An inter-island comparison of CORT secretion (for four populations) revealed that baseline CORT secretion was similar among populations during the non-breeding and breeding seasons; however, the CORT response to capture-restraint varied significantly among populations. Inter-population variation in testosterone (T) was

observed in males (but not females) and was positively linked with increased baseline CORT from the non-breeding season to the breeding season, suggesting male reproductive activity may drive seasonal change of baseline CORT. Significant correlations were observed between the CORT response to capture-restraint (but not baseline CORT) and habitat elements of latitude, tuatara density and seabird abundance and 2) demogenetic factors of sex ratio and genetic diversity. The measurement of CORT as a physiological monitoring tool indicated that short- and long- term dynamics of CORT secretion in tuatara are not altered through translocation to a new island, as the acute CORT response remained stable throughout exposure to cumulative stressors and long-term dynamics of CORT secretion in translocated populations simultaneously mirrored those in source populations.

These findings deliver the most detailed study of CORT secretion patterns in tuatara to date. Moreover, as the first study to apply CORT secretion data as an conservation physiology monitoring tool in tuatara, these results serve as a baseline reference for future research and monitoring of conservation efforts.

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To my mom, Linda

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CHAPTER 1

Biology of glucocorticoids and their application in conservation: an overview

1.1 Introduction

Human impacts continue to influence ecological change of natural ecosystems; therefore, it is becoming increasingly important to understand how organisms respond and cope with environmental change and unpredicted challenges (Wingfield, 2013). Physiological techniques have been utilised in ecological/conservation studies as tools to assess an individual's physiological response to challenges or a changing environment (Wikelski and Cooke, 2006). Understanding an individual's physiological response to certain challenges or stressors, and/or identifying the root of physiological stress, will enable development and consideration of more effective conservation management strategies and solutions (Dantzer *et al.*, 2014; Madliger and Love, 2014). This thesis aims to advance the current understanding of reptile physiology by identifying intrinsic and extrinsic factors associated with the endocrine stress response in a rare reptile, the tuatara (*Sphenodon punctatus*), and subsequently examine its utility in monitoring conservation efforts. In this overview, I provide a brief background of glucocorticoid physiology, conservation physiology, and tuatara ecology to provide a general introductory framework for the four data chapters that follow.

1.2 Glucocorticoids: physiology and function in vertebrates

The endocrine system in vertebrates produces hormones that regulate and facilitate life-history transitions and responses to intrinsic and extrinsic changes/challenges

(i.e. stressors), thereby playing an important fitness role (Crespi *et al.*, 2013; Dufty *et al.*, 2002; Jessop *et al.*, 2013; Schultner *et al.*, 2013). The hypothalamo-pituitary-adrenal (HPA) axis produces steroid hormones referred to as glucocorticoids (GCs) (Fig 1.1). At a basal level, GC secretion promotes essential life processes such as carbohydrate/ intermediary metabolism balance and cell provisioning of glucose (i.e. basic energy regulation). However, when an individual is exposed to unpredictable challenges/stressors, a complex adaptive hormonal response commonly referred to as the 'stress response' cascades through the HPA axis and stimulates further secretion of GCs (Busch and Hayward, 2009; Landys *et al.*, 2006; Sapolsky *et al.*, 2000)(Fig 1.1).

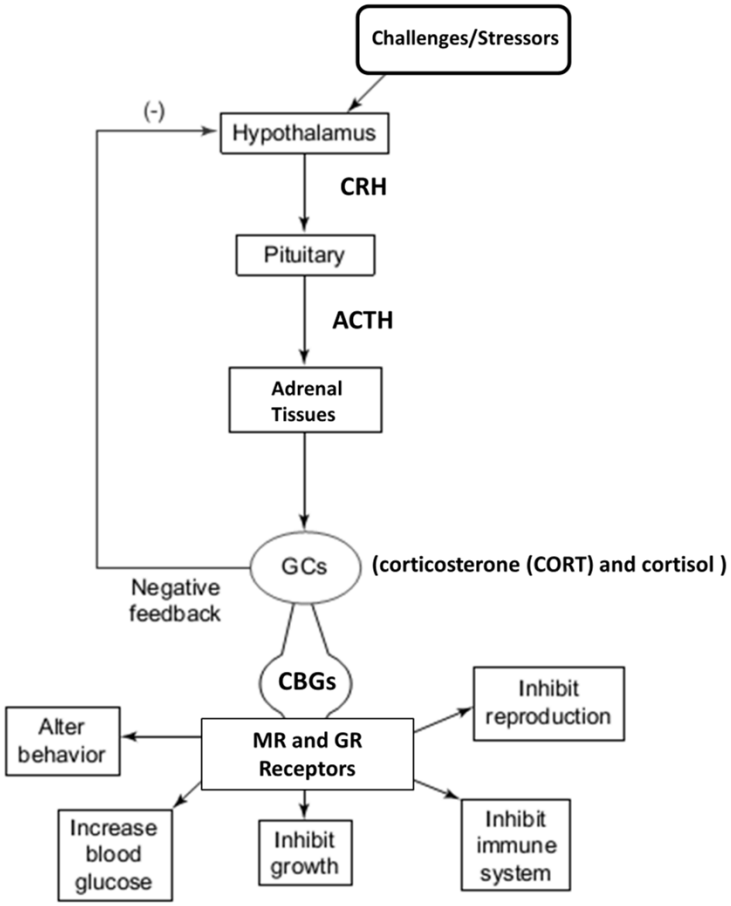

Figure 1.1. The vertebrate hypothalamo-pituitary-adrenal (HPA) axis. Increased glucocorticoid (GC) secretion (corticosterone (CORT) and cortisol) is activated through stimulation of the HPA axis in response to challenges/stressors. CORT is dispersed through the circulatory system by way of corticosteroid-binding globulins (CBGs). Figure modified from Romero (2004).

The hypothalamus is stimulated in response to a challenge or stressor, triggering release of corticotropin-releasing hormone (CRH). In turn, CRH stimulates the pituitary to synthesise and release adrenocorticotropin hormone (ACTH), which then stimulates adrenal tissues to increase GC secretion well above basal levels (Sheriff *et al.*, 2011) (Fig 1.1).

The primary GCs in vertebrates are corticosterone (CORT) and cortisol. CORT is dispersed through the circulatory system by way of corticosteroid-binding globulins (CBGs), and is delivered to specific target tissues, whereupon CORT binds to receptors (Schoech *et al.*, 2013)(Fig 1.1). Receptors are widely distributed throughout cells in the body, and can therefore initiate a range of responses affecting behaviour, energy provision by stimulating gluconeogenesis (the formation of "new" glucose from non-carbohydrate sources), growth, immune response, and reproduction (Jessop *et al.*, 2013; Romero, 2004) (Fig 1.1). CORT is modulated by two types of receptors: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). At lower CORT levels (i.e. basal CORT secretion), CORT preferentially binds to MRs (to regulate energy balance) and in contrast, GR receptors are bound when CORT concentrations are high (i.e. after a challenge/stressor) (Busch and Hayward, 2009; Romero, 2004). A negative feedback cycle regulates the continuation (or conversely discontinuation) of the CORT secretion process, and is based on the prevalence/status of the challenge or stressor (Romero, 2004; Sapolsky *et al.*, 2000) (Fig 1.1).

Although GCs primarily function to support immediate survival and fitness, elevated GCs over extended periods of time (i.e. chronic stress) have been shown to have deleterious effects on an individual's overall health, condition and reproductive success (Tokarz and Summers, 2011). Harmful effects of elevated or sustained CORT secretion (both endogenous and exogenous) have been confirmed in a wide range of taxa. Examples include: decreased body condition in brown tree snakes (*Boiga irregularis*) (Waye and Mason, 2008), reduced bacterial killing ability and slower wound healing in marine iguanas (*Amblyrhynchus cristatus*) (French *et al.*, 2010), reduced display and attack behaviour (DeNardo and Licht, 1993) and home

range territories (DeNardo and Sinervo, 1994) in side-blotched lizards (*Uta stansburiana*).

Several studies have also shown that maternal CORT is transferred to young (in both viviparous and oviparous species) (Cree *et al.*, 2003; Love *et al.*, 2013; Uller *et al.*, 2009). Hormones contribute to developmental plasticity in individuals and reproducing females exposed to stressors can affect morphological, physiological and behavioural traits in offspring that can subsequently affect their performance (Meylan *et al.*, 2010; Michel *et al.*, 2011). For example, stressed barn swallows (*Hirundo rustica*) (exposed to predators) laid eggs that had higher CORT than un-stressed controls (exposed to herbivores) and eggs with higher CORT had lower hatching success and produced smaller hatchlings with slower plumage development, compared to controls (Saino *et al.*, 2005). Pregnant female garter snakes (*Thamnophis elegans*) with elevated CORT produced offspring that exhibited decreased anti-predator behaviour (Robert *et al.*, 2009). Activity (Belliure *et al.*, 2004) and sprint speed/motivation to run (Meylan and Clobert, 2004) were decreased in common lizard (*Lacerta vivipara*) hatchlings produced by mothers with elevated CORT. Recent studies also demonstrate that CORT can influence sex determination of embryos. For example, in two lizard species that have temperature-dependent sex determination (TSD), elevated CORT altered sex-ratios in offspring; more males were produced in *Bassiana duperreyi* and more females were produced in *Amphibolurus muricatus* at pivotal temperatures (normally producing a 1:1 sex ratio). Sex-specific embryonic mortality was affected by CORT levels in *A. muricatus,* but not in *B. duperreyi* (Warner *et al.*, 2009).

1.3 Factors associated with CORT secretion

It is generally agreed that baseline CORT serves to meet the energetic demands associated with everyday life, whereas the acute CORT response is a reaction to unpredictable and/or challenging events (Fig 1.2) (Tokarz and Summers, 2011; Wingfield *et al.*, 1998). The acute CORT response allows individuals to react to a stressor by way of an adaptive physiological and/or behavioural response, and serves to promote overall fitness (Breuner *et al.*, 2008; Busch and Hayward, 2009).

Modulation of CORT secretion (i.e. daily and/or seasonal)(Fig 1.2) occurs and helps individuals cope with their specific environment to effectively increase fitness (Moore and Jessop, 2003; Romero, 2002).

Baseline CORT is commonly elevated during the breeding season to support energetic requirements associated with reproductive activities (such as nesting and mating), and similarly, individuals experiencing reduced breeding opportunities or living in harsh environments have been found to dampen their CORT response during the breeding season, which may serve to increase chances of reproductive success (Ebensperger *et al.*, 2013; Holding *et al.*, 2014b; Moore and Jessop, 2003; Romero, 2006; Wingfield *et al.*, 1998; Wingfield and Sapolsky, 2003). For example, sea turtles display a reduced CORT response during nesting, even when faced with challenges such as non-lethal shark attacks and overheating, suggesting a trade-off of immediate survival for fitness (Jessop *et al.*, 2004a; Jessop *et al.*, 2000).

Patterns of CORT secretion are not universally consistent; variation is observed among species and within/among populations (Baker *et al.*, 2013; Cockrem, 2013; Creel *et al.*, 2013; Love *et al.*, 2013). Variation in CORT secretion can be linked with intrinsic factors such as sex, reproductive status, body condition, and age (reviewed in Baker *et al.* (2013) and Cockrem (2013)). Also, extrinsic factors such as habitat modification – both natural and human-induced (Berner *et al.*, 2013; French *et al.*, 2010; Owen *et al.*, 2014; Tartu *et al.*, 2014; Taylor *et al.*, 2014), seasonal/annual changes in food availability (Bryan *et al.*, 2014; Kitaysky *et al.*, 2007; Woodley *et al.*, 2003), and fluctuating environmental temperature and/or weather (Lance *et al.*, 2010; Telemeco and Addis, 2014) can influence CORT secretion. Furthermore, increased energy demands required during reproduction (Woodley and Moore, 2002), territorial disputes (Baird *et al.*, 2014; Yang and Wilczynski, 2003) and migration (Hamann *et al.*, 2007; Jessop *et al.*, 2004b) can influence patterns of CORT secretion.

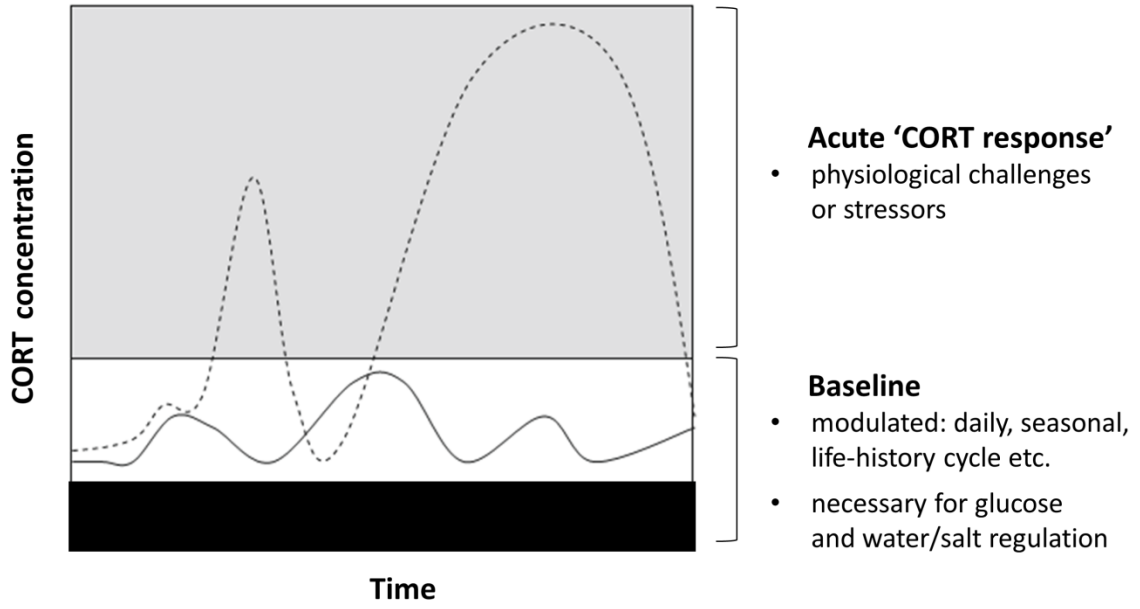

Figure 1.2. Schematic diagram of CORT secretion in vertebrates. Baseline CORT concentrations reflect the basal range necessary for basic life functions (black band) and modulated range in response to daily, seasonal and life-history demands (solid black line within white band). An acute CORT response (dashed line), in which CORT concentrations are significantly elevated from baseline, will be mounted in response to physiological challenges or stressors. Figure modified from Busch and Hayward (2009).

1.4 Corticosterone as an indicator of physiological stress

The measurement of CORT as a tool to assess population health and/or to monitor conservation efforts is instrumental in stress physiology research, with CORT being the most widely used indicator of physiological stress in vertebrates (Busch and Hayward, 2009; Dantzer *et al.*, 2014; Wikelski and Cooke, 2006). Increased CORT secretion in vertebrates has been linked with reduced body condition, depressed immunity, decreased locomotor performance, and changes in behaviour - all of which are performance measures that can have fitness implications (Busch and Hayward, 2009; DeNardo and Sinervo, 1994; Meylan and Clobert, 2004; Towns *et al.*, 2007; Warner *et al.*, 2009; Waye and Mason, 2008).

Applied conservation physiology has been used to monitor and/or assess issues and conservation efforts such as: habitat alteration/human exposure (Owen *et al.*, 2014; Romero and Wikelski, 2002; Taylor *et al.*, 2014), weather and climate change (Breuner and Hahn, 2003; Lance *et al.*, 2010; Thierry *et al.*, 2013), increased predation and/or invasive species (Anson *et al.*, 2013; Berger *et al.*, 2007; Rödl *et al.*, 2007; Thaker *et al.*, 2010), immune function and disease (Lucas and French, 2012; Martin *et al.*, 2012), reproduction (Klose *et al.*, 2006; Moore *et al.*, 2005; Smith *et al.*, 2012; Strasser and Heath, 2013; Tartu *et al.*, 2014), and translocations (Bosson *et al.*, 2013; Drake *et al.*, 2012; Franceschini *et al.*, 2008; Holding *et al.*, 2014a; Socorro Aguilar-Cucurachi *et al.*, 2010; Zidon *et al.*, 2009).

For example, Romero and Wikelski (2001, 2010) determined CORT secretion in Galápagos marine iguanas (*Amblyrhynchus cristatus*) during El Niño and found that 1) CORT secretion (both baseline CORT and the CORT response to capture-restraint) was higher during El Niño-induced famine conditions (compared to non-famine conditions), 2) baseline CORT was elevated in individuals with body condition scores below a certain threshold, and 3) the CORT response to capture-restraint and negative feedback of CORT predicted survival. Hartup *et al.* (2005) examined CORT secretion (faecal CORT concentrations) to assess potential stress in whooping cranes (*Grus americana*) undergoing translocation to the wild. The authors found that acute stressors of capture, restraint, and severe weather were connected with CORT secretion, but the overall translocation process (i.e. costume-rearing, ultralight aircraft habituation, training, artificial migration) did not lead to chronic stress.

There is still confusion, however, in how to identify chronic stress in wild animals, although it is commonly assumed that measures of CORT will increase. The best approach for identifying a chronically stressed population appears to be documentation of changes in CORT secretion, as the detection of a change in response may be more important than the direction of change (increase or decrease) (Dantzer *et al.*, 2014; Dickens and Romero, 2013).

1.4.1 Determining CORT concentrations

1.4.1.1 Immunoassay

Corticosterone concentrations are determined in biological samples (i.e. blood plasma/serum, saliva, urine, faeces, hair, feather, skin, milk, and albumen/yolk) mainly by either enzyme-immunoassay (EIA) or radio-immunoassay (RIA) (Sheriff *et al.*, 2011). Both EIA and RIA techniques use competitive binding assays that are extremely sensitive in detecting CORT. Assays can be developed in-house or be purchased as commercial kits (complete with all necessary components). Both options need to be carefully validated for the species and biological sample of choice (as in Chapter Two) to ensure proper quantification of CORT concentrations, as other variables can interfere with the assay and the validation process will identify any issues (Sheriff *et al.*, 2011).

1.4.1.2 Total vs free CORT in blood plasma samples

Determining CORT concentrations in blood samples (by assaying plasma or serum) is the most commonly used method in vertebrate studies. Blood samples provide an instantaneous 'snap-shot' of circulating CORT concentrations in an individual, for both baseline and CORT response measures (Sheriff *et al.*, 2011) (Fig 1.2). Assays of plasma/serum CORT measure the total available CORT concentration (versus free CORT concentration – unbound to CBGs) in a blood sample. Breuner *et al.* (2013) recommended that researchers determine and report both total CORT and free CORT concentrations, arguing that free CORT is the biologically relevant component in blood. However, total CORT concentrations are currently considered more accurate and more interpretable/relevant for ecological field studies than free CORT concentrations, as total CORT indicates the total available CORT that will eventually be utilised by tissues (Schoech *et al.*, 2013).

1.5 Tuatara ecology

The Tuatara (*Sphenodon punctatus*) (Fig 1.3) is a protected reptile species endemic to New Zealand and the sole living representative of the order Rhynchocephalia, the sister group to squamates (Cree, 2014). Tuatara are sexually dimorphic once they reach sexual maturity at approximately 13 years of age (~ SVL ≥ 170mm), whereupon sex can be identified by examining characteristics such as head size,

head shape, body shape, crest development and spine shape (Cree, 2014; Cree *et al.*, 1991a; Dawbin, 1982). Tuatara have seasonally distinct breeding and nesting seasons, with breeding activity (mating and ovulation) occurring in autumn (February – March) and nesting activity occurring in spring (October – December), though not all females ovulate and nest each year (Cree, 2014). Female tuatara exhibit an extended reproductive cycle, carrying eggs in the oviduct for 6–8 months and producing one clutch of eggs every 2-9 years, depending on the population (Cree *et al.*, 1992; Moore *et al.*, 2009a; Refsnider *et al.*, 2010).

Historically, tuatara were widely distributed throughout New Zealand, but following human settlement, they became extinct on the mainland (North and South Islands). Currently, natural populations occur on 32 isolated offshore islands and translocated populations occur on nine offshore islands and five mainland sanctuaries (Cree, 2014; Jones and Cree, 2012). Captive breeding programs, eradication of invasive species/predator control, and translocations (Cree, 2014; Gaze, 2001) are relevant ongoing conservation efforts for tuatara, all of which have potential to benefit from relevant endocrine data.

Figure 1.3. A wild adult male tuatara (*Sphenodon punctatus*) that was hand-captured on Taranga Island, and subsequently sampled by Lindsay Anderson. Photo: Susan Keall.

Previous research has shown that baseline CORT in tuatara is generally low, with no evidence of a diel cycle (Cree and Tyrrell, 2001; Tyrrell and Cree, 1998), and a significant CORT response is observed to capture-restraint (Tyrrell and Cree, 1998). Seasonal variation in baseline CORT has been observed in the population on Stephens Island/Takapourewa, where baseline CORT was highest in November for females and February and May for males (Tyrrell and Cree, 1998); however, seasonal patterns of baseline CORT secretion in other island populations, seasonal patterns of the CORT response, and factors influencing inter-island variation have not been investigated. Overall, we know very little about how intrinsic and extrinsic factors influence variation in CORT secretion (both baseline CORT and the CORT response) within and among populations of tuatara.

1.6 Study populations/sites

The study populations/sites (Fig 1.4) in this thesis were chosen based on population size and connection to ongoing conservation efforts. Stephens Island has the largest population of tuatara, and was chosen as the study site to thoroughly examine intrinsic factors influencing CORT secretion (baseline CORT and a 24 h CORT response time-series) in male and female (both gravid and non-gravid) tuatara (Chapter Two). The Stephens Island, North Brother Island, Lady Alice Island, and Taranga Island populations were included in studies examining the influence of temperature on CORT secretion (Chapter Three) and inter-island variation of CORT secretion (Chapter Four). Finally, Stephens Island and Lady Alice Island were source populations in two translocation programmes (Chapter Five), where I examined CORT secretion through the translocation process in the translocated populations of Motuihe Island (from Lady Alice Island), Sanctuary Mountain Maungatautari and Cape Kidnappers Sanctuary (both from Stephens Island).

Figure 1.4. Location of study populations/sites mentioned in this thesis. Naturally occurring island populations are indicated by black dots and translocated populations are indicated by white dots.

1.7 Thesis structure

The data chapters of this thesis are written as stand-alone manuscripts that have been submitted to or published in peer-reviewed journals. Therefore, there is inevitably some repetition, specifically in the introduction and methods sections (note: references for all chapters are combined at the end of this thesis). I led the study design, data collection, data analyses, and writing of this thesis, under the guidance and supervision of my supervisors who are listed as co-authors for all four data chapters.

The overall aim of this thesis is to advance the understanding of CORT physiology in the tuatara (*Sphenodon punctatus*) and to assess the value of monitoring CORT in relevant conservation efforts. The results from this thesis will contribute to the broader field of comparative endocrinology and will provide applied results and information to conservation managers and future researchers.

The main questions of this thesis are:

1) What factors influence CORT secretion (both baseline CORT and the CORT response) in tuatara?

2) Do patterns of CORT secretion indicate a physiological response to challenges/stressors in tuatara?

3) Is the measurement of CORT secretion a valuable addition to the conservation 'tool-box' for tuatara?

Basic intrinsic patterns of CORT secretion must first be understood in order to reliably interpret and/or compare outcomes from a study employing CORT secretion as a tool. Failing to account for influential factors and correlates of CORT secretion can lead to uncertain results. Therefore, in Chapter Two, I conduct a 24 h time-series of capture-restraint in a wild population of tuatara on Stephens Island. I simultaneously examine patterns of CORT secretion in males, non-gravid females, and gravid females. I test the hypothesis that gravid females have a dampened CORT response to capture-restraint compared to non-gravid females. I also consider correlations with body condition and internal body temperature. Chapter Two has been published as:

> Anderson L, Cree A, Towns D, Nelson N (2014) Modulation of corticosterone secretion in tuatara (*Sphenodon punctatus*): evidence of a dampened stress response in gravid females. General and Comparative Endocrinology 201: 45-52

Chapter Three expands on results from Chapter Two, to further examine (both observationally and experimentally) the correlation between internal body temperature (T_b) and CORT secretion. I test the hypothesis that an acute increase in T_b increases the CORT response to capture-restraint. Chapter Three has been submitted to Physiological and Biochemical Zoology as:

> Anderson L, Nelson N, Cree A (*in review*) Increased body temperature amplifies the corticosterone stress response in tuatara (*Sphenodon punctatus*). Physiological and Biochemical Zoology

Taken together, Chapters Two and Three identify key intrinsic factors that are correlated with baseline CORT and the CORT response in tuatara. Chapters Four and Five build on (and consider) results from the previous chapters to examine if patterns of CORT secretion indicate a physiological response to challenges/stressors and assess the value of CORT measurement as a conservation physiology tool in tuatara conservation efforts.

Chapter Four compares CORT secretion among four contrasting island populations (with respect to potential challenges/stressors) and identifies ecological attributes that best explain variation in CORT secretion among populations. I test the hypotheses that (i) baseline CORT varies among populations, (ii) baseline CORT is higher during the breeding season, compared to the non-breeding season, and (iii) the CORT response is higher in northern populations that experience a milder climate and past/recent presence of an introduced mammal, the Pacific rat (*Rattus exulans*). Chapter Four has been submitted to Animal Conservation as:

> Anderson L, Nelson N, Towns D, Cree A (*in review*) The corticosterone stress response varies among island populations of tuatara (*Sphenodon punctatus*) and is predicted by linear ecological attributes. Animal Conservation.

Chapter Five monitors the CORT response through the initial translocation procedure in tuatara, and examines long-term patterns of CORT secretion in

translocated populations as an indicator of chronic stress. I test the hypothesis that cumulative stressors (experienced during the translocation process) and translocation to a novel location increase CORT secretion. Chapter Five has been published as:

> Anderson L, Cree A, Towns D, Nelson (2015) Moving house: Long-term dynamics of corticosterone secretion are unaltered in translocated populations of a rare reptile (the tuatara, *Sphenodon punctatus*). Conservation Physiology. DOI: 10.1093/conphys/cov014

Chapter Six summarises the main findings of this thesis, discusses conservation applications and the value of measuring CORT secretion as a conservation physiology tool in tuatara, and identifies knowledge gaps and areas for future research.

CHAPTER 2

Modulation of corticosterone secretion in tuatara (*Sphenodon punctatus*): Evidence of a dampened stress response in gravid females[1]

2.1 Abstract

Baseline and stress response glucocorticoid (GC) secretion can be modulated by individuals to support activities and physiological functions connected with reproduction (migration, mating, oviposition and/or parturition, care of young). Corticosterone (CORT) is the primary GC in reptiles and, in accordance with other vertebrates, an adrenocortical stress response is observed. Modulation of CORT secretion occurs in several reptile species, such that elevated baseline CORT and/or a dampened CORT response are common during reproductive life-history events. I investigated CORT secretion after 24 h capture-restraint in the oviparous tuatara (*Sphenodon punctatus*), the last living rhynchocephalian, and tested whether gravid females have a dampened CORT response compared with non-gravid females. I also included males as a comparison. I confirmed that gravid females have significantly higher baseline plasma CORT than non-gravid females, suggesting increased CORT secretion during nesting. Furthermore, I found that gravid females exhibit a dampened CORT response compared to non-gravid females and males. My results demonstrate that female reproductive condition is correlated with CORT secretion in tuatara, and suggest that CORT secretion is modulated in gravid females during

[1] This chapter is based on the following publication with minor modifications:
Anderson L, Cree A, Towns D, Nelson N (2014) Modulation of corticosterone secretion in tuatara (*Sphenodon punctatus*): evidence of a dampened stress response in gravid females. General and Comparative Endocrinology 201: 45-52

nesting to maintain homeostasis, which could increase chances of reproductive success and/or promote overall fitness.

2.2 Introduction

Animals continuously adjust to their environment to support immediate survival and long-term fitness. Environmental perturbations such as increased predator abundance, decreased food availability, habitat alteration, human exposure, pollution and extreme weather can test individuals' abilities to maintain homeostasis (Angelier and Wingfield, 2013; Busch and Hayward, 2009; Langkilde and Trompeter, 2011; Romero and Wikelski, 2001; Wingfield and Sapolsky, 2003). Similarly, physiological and social demands such as reproduction, migration and territorial disputes can be challenging (Creel et al., 2013; Klukowski, 2011; Phillips and Klukowski, 2008; Yang and Wilczynski, 2003). One way that individuals manage these environmental, social and physical challenges is through release of glucocorticoids (GCs) from adrenal tissues.

GCs promote basic life processes such as energy intake and cell provisioning of glucose (Landys *et al.*, 2006). When individuals are subject to challenging stimuli, an adaptive integrated process commonly referred to as the 'stress response' activates the hypothalamo-pituitary-adrenal (HPA) axis and leads to an increase in GC secretion (Boonstra, 2013; Busch and Hayward, 2009; Cockrem, 2013). This surge of GCs (along with other related hormones, signalling molecules and complementary processes) mobilises and diverts energy resources towards muscular, cardiovascular and cognitive systems to promote immediate survival, a reaction that is conserved across the majority of vertebrate groups (Cockrem, 2013; Sapolsky *et al.*, 2000; Wingfield and Sapolsky, 2003). Although GCs primarily function to support immediate survival and fitness, elevated GCs over extended periods of time (i.e. chronic stress) have been shown to have deleterious effects on an individual's overall health, condition and reproductive success (Almasi *et al.*, 2013; Sapolsky *et al.*, 2000). Nevertheless, baseline and stress response GC secretion can be modulated by individuals to support activities and physiological functions connected

with reproduction (migration, mating, oviposition and/or parturition, care of young).

Glucocorticoid modulation during reproductive life-history stages is attributable to higher energetic demands and serves to maintain homeostasis and promote reproductive success (Breuner *et al.*, 2008; Love *et al.*, 2008; Romero, 2002). Therefore, identifying and understanding modulation of GCs and the GC stress response in a context-dependent manner clarifies relationships between GC secretion, behaviour and potential fitness trade-offs (Angelier and Wingfield, 2013; Busch and Hayward, 2009; Madliger and Love, 2014).

Research investigating synthesis, secretion and modulation of GCs has focussed primarily on fish, mammals and birds, with reptiles and amphibians constituting only ~ 10% of studies to date (Baker *et al.*, 2013; Bonier *et al.*, 2009; Breuner *et al.*, 2008; Cockrem, 2013). Reptiles are good model organisms to examine modulation of GC secretion as they exhibit a diverse range of characteristics (oviparity/viviparity, several different sex-determining patterns, various reproductive systems and life-history strategies) conducive to studying the interplay and potential trade-offs between stress, reproductive success and survival (Crews and Moore, 1986; Moore and Jessop, 2003). In reptiles, the primary GC is corticosterone (CORT), and similar to other vertebrates a CORT response to stressors is observed (Cockrem, 2013; Romero, 2002). Modulation of CORT secretion occurs in several reptile species, such that elevated baseline CORT concentration and/or a dampened CORT response are common during reproductive life-history events (Moore and Jessop, 2003; Wingfield and Sapolsky, 2003). The functional significances and underlying mechanisms of this trait in gravid/pregnant reptiles are unclear, but some authors have suggested potential links with egg/embryo maintenance (Cree and Tyrrell, 2001), with adaptive maternal effects such as shielding embryos from hormone exposure or influences on offspring phenotype (Cartledge and Jones, 2007; Uller *et al.*, 2009; Warner *et al.*, 2009), and with timing of oviposition/parturition (Jones and Guillette, 1982; Smith *et al.*, 2012).

When breeding individuals are exposed to stressors, a dampened CORT response may also serve to buffer activation of the 'emergency life-history stage ' (in which immediate survival is prioritized and activities such as mating and nesting attempts can be abandoned) to increase chances of successful reproduction (Jessop *et al.*, 2000; Moore and Jessop, 2003; Wingfield *et al.*, 1998; Wingfield and Sapolsky, 2003). For example, several species of sea turtle show a dampened CORT response during nesting, even when faced with additional (potentially lethal) stressors such as extreme predation attacks and overheating, suggesting a trade-off of immediate survival for an increased chance of reproductive success (Jessop *et al.*, 2004a; Jessop *et al.*, 2000). Lack of a significant CORT response during breeding events could also suggest an inability to mount a response due to changes in the capacity of the adrenal gland to secrete CORT (Romero, 2006).

Numerous intrinsic and extrinsic factors can influence modulation of baseline CORT concentration and the CORT response in reptiles. These include age, sex, reproductive condition, body condition, season, weather, pathogens and population (Baker *et al.*, 2013; Boonstra, 2013; Breuner *et al.*, 2008; Creel *et al.*, 2013; Eikenaar *et al.*, 2012; Wingfield, 2013). In recent reviews of the existing literature, it is apparent that failing to account for influential factors and correlates of CORT secretion can lead to uncertain results (Baker *et al.*, 2013; Busch and Hayward, 2009; Cockrem, 2013; Creel *et al.*, 2013; Wingfield, 2013). For example, comparing modulation of CORT secretion in breeding and non-breeding reptiles is often confounded with seasonal changes, as sampling of non-breeding individuals takes place outside of the breeding season (for both males and females) (Cartledge and Jones, 2007; Klukowski, 2011; Moore and Jessop, 2003; Selman *et al.*, 2012). Variation between geographically separated populations (both permanently and temporarily) can also be a limiting factor. For example, in a study comparing the CORT response in breeding and non-breeding female green sea turtles (*Chelonia mydas*), season was controlled for but non-breeding females were part of a permanently resident reef population whereas breeding females were migratory non-residents (Jessop *et al.*, 2000).

To my knowledge, only two studies have simultaneously investigated the modulation of CORT secretion in reptiles, in a way that allowed for separation of the effects of sex, reproductive condition, season and population. For example, the viviparous New Zealand common gecko (*Woodworthia maculatus*) and oviparous tree lizard (*Urosaurus ornatus*) are model reptile species that allow for concurrent sampling of gravid female, non-gravid female and male individuals, without confounding factors such as population and season (Cree *et al.*, 2003; Woodley and Moore, 2002). Interestingly, modulation of the CORT response was not observed in pregnant female common geckos; therefore, the functional significance and mechanism of the ability to modulate CORT secretion (with regards to female reproductive condition) in reptiles may not be fruitful to be explored further in this species (Cree *et al.*, 2003). A dampened CORT response was observed in gravid female tree lizards, compared with vitellogenic females and males (Woodley and Moore, 2002). However in gravid females, an obvious temporal separation of mating, ovulation, and nesting/oviposition activity is lacking (as oviposition occurs ~ 1 week after ovulation), making it difficult to tease out the functional significance of CORT modulation (with regards to gravid females) in this species.

Tuatara (*Sphenodon punctatus*) are an attractive model species for studies investigating CORT secretion and modulation as their distribution and biology allows for simultaneous control of factors that can influence CORT release. Here, I test for a dampened CORT response in gravid females of this oviparous species during the nesting life-history stage. Tuatara are a protected reptile species endemic to New Zealand, and wild populations are currently restricted to isolated offshore islands (Jones and Cree, 2012). They are the only extant representatives of the order Rhynchocephalia, which is the sister group to squamates (Jones, 2008). Studying tuatara could therefore contribute to understanding of patterns that are general among amniotes, for example whether nesting in oviparous species is associated with a dampened CORT response to capture. Tuatara reach sexual maturity at approximately 13 years of age (~ SVL ≥ 170 mm), and males and females can be identified by examining secondary sex characteristics such as head size/shape, body shape, crest development and spine shape (Cree *et al.*, 1991a; Dawbin, 1982). Tuatara have a seasonally distinct reproductive cycle with mating

taking place in the austral autumn (February – March) and nesting in the austral spring (October – November). Females do not have an annual reproductive cycle; instead, ovulation and nesting events occur only every 2-9 years (Cree *et al.*, 1992; Moore *et al.*, 2009a; Refsnider *et al.*, 2010). Therefore, gravid and non-gravid females can be found in the same population at the same time, allowing for control of reproductive condition and season. Nesting is separated by 6-7 months from mating and ovulation in tuatara (Cree, 1994); therefore, patterns in CORT secretion that are related specifically to nesting/oviposition are much more readily identified than in other reptiles.

Baseline CORT secretion in tuatara is relatively low and variation is observed seasonally, but not diurnally (Tyrrell and Cree, 1998). Gravid females have significantly higher baseline CORT levels during the nesting life-history stage compared with non-gravid females and males, with levels highest during nest digging and oviposition (Cree and Tyrrell, 2001). Interestingly, a distinct fall (~5-fold) in baseline CORT secretion is observed after oviposition, even in the case of females that continue to guard their nests, which suggests a potential role of CORT in the timing of oviposition (Cree and Tyrrell, 2001). In accordance with other vertebrate species, a stress response to capture-restraint is detected in tuatara, although CORT concentration values at 3 h capture-restraint, are relatively low compared to other reptiles (Tyrrell, 1993; Tyrrell and Cree, 1998). Studies examining the magnitude and duration of the CORT response are limited (Cree and Tyrrell, 2001), and CORT modulation in gravid females during the nesting life-history stage has not been examined.

The aim of this study was to test whether gravid females exhibit a modulated CORT response during the nesting life-history stage compared with non-gravid females. Males were included for comparison. By examining the responses of gravid females, non-gravid females and males simultaneously, I am able to identify variation in the CORT response related to sex and reproductive condition, apart from seasonal or environmental changes. I also tested for interactions between the CORT response and predictors such as body temperature and body condition, as some studies report a positive relationship between internal body temperature and baseline

CORT concentration (including studies in tuatara) (Cree *et al.*, 1990a; Cree *et al.*, 2003) and a negative relationship between body condition and magnitude of the CORT response (Baker *et al.*, 2013; Dunlap and Wingfield, 1995).

2.3 Material and methods

2.3.1 Study animals and experimental design

Wild adult tuatara (*Sphenodon punctatus*) were captured and sampled on Stephens Island/Takapourewa (40° 40' S, 174° 00' E) in Cook Strait, New Zealand during the 2011 nesting season (October – December, austral spring). Individuals that had emerged from burrows were caught by hand at night between 20:00 h and 05:00 h and were subsequently assigned to an appropriate group based on sex and reproductive status (male, gravid female, or non-gravid female). Gravid females were selectively captured at or near a nesting rookery, and female reproductive status was inferred through abdominal palpation for shelled eggs. Following the blood sampling described in section 2.3.2, synthetic oxytocin (10 I.U./ml/kg body mass) (Thompson *et al.*, 1991) was administered by an intraperitoneal injection to a subset of gravid females (n=13) (as a component of another study - unpublished). Oviposition was observed within 24 h in 12 of the 13 females, which helped validate the inference of reproductive status from palpation.

2.3.2 CORT response to 24 h capture-restraint

Male (n=30), gravid female (n=28) and non-gravid female (n=31) tuatara were subject to a standardized capture-restraint protocol to determine patterns of CORT secretion over time. Capture/sampling occurred over 2 nights during the 2011 nesting season (25–26 October). To determine baseline CORT levels, a blood sample (up to 1 ml) was collected within 10 min of capture (mean = 5.22 ± 0.34 min) from the base of the tail with a heparinized 23-gauge needle and 1 ml syringe. Previous studies of tuatara observed no significant effect of bleeding effort (time from capture to blood sample collection) on baseline CORT concentration in blood samples collected within 20 min of capture (Tyrrell, 1993). As expected, I observed no correlation between duration of bleeding effort and baseline CORT concentration for

samples obtained within 10 min of capture (r=-0.085, P=0.429). After baseline samples were taken, individuals were randomly assigned to capture-restraint treatment times of 1.5, 3, 6, 12, 18 or 24 h (n=4-5/group/treatment time) to determine the 24 h CORT response and were held in a cloth capture bag for assigned times in a quiet room inside the research house (basic accommodation on Stephens Island). A second blood sample (up to 1 ml) was taken from each individual immediately after the assigned capture-restraint treatment time was reached. Individuals were randomly selected to obtain desired sample sizes and were sampled for blood only twice in 24 h. Blood samples were separated into plasma and red blood cell components under normal gravity (as centrifugation was not available at the remote island field site) for 6 to 8 h at 4°C (CORT levels in plasma and serum have been shown to remain stable when kept at room temperature and 4°C for up to 72 h, with and without centrifugation)(Reimers *et al.*, 1983; Sheriff *et al.*, 2011). Plasma was transferred into cryogenic vials with a micropipette and held in a freezer at -20°C until returned to the laboratory where it was stored at -80°C until assayed.

2.3.3 Internal body temperature and body-condition measurements

Internal body temperature (T_b) was recorded with a cloacal thermocouple (Fluke® Multimeter, model: 179, specified accuracy ±0.1°C, USA) prior to taking blood samples for each individual (both baseline and stress response). Morphometric measurements were taken after capture-restraint and CORT response blood sampling was completed. Individual animals were weighed to the nearest ± 5 g with a 1000 g spring scale (Pesola AG, Switzerland) to determine mass (g), and snout-vent length (mm), tail length (mm), tail regeneration length (mm), and pelvis width (mm) were measured with a straight ruler. I calculated tail-corrected mass following the equation described by Newman *et al.* (1994) to account for tail-loss and regeneration (Newman *et al.*, 1994) . I pooled morphometric measurement data (male, non-gravid, gravid) to generate individual body-condition scores using principal components analysis (PCA) to incorporate size-index traits related to condition (SVL, width, mass) (Taillon *et al.*, 2011). This created a correlation matrix that produced a 'size-index' scoring system in which the first principal component (PC1) explained 84.9% of the total variance. For this study, PC1 scores are

considered to be a measure of tuatara body condition. The body-condition score (PC1) was dominated by the following morphometric loadings: tail-corrected mass (TCM), pelvis width (PW) and snout-vent length (SVL). The scoring system equation for PC1 is 0.602TCM + 0.534PW + 0.593SVL, and as all loadings have positive coefficients this indicates that individuals with greater measurements of TCM, PW and SVL will have higher body-condition scores. This scoring system was compared with an alternative scoring system (standardized residuals from the regression of TCM on SVL) and a strong correlation was observed (r=0.981, P<0.001).

2.3.4 Hormone determination and enzyme immunoassay (EIA) validation

Plasma samples were randomly selected for assay (29/plate), thawed at room temperature and spun in a centrifuge at 5000 rpm for 5 min at 4°C to separate any residual debris. Aliquots of 10 µl plasma were extracted once with 600 µl of freshly distilled dichloromethane. After 15 min of incubation at room temperature, samples were snap-frozen in a -70°C ethanol bath, decanted into clean glass vials, and dried in a vacuum oven at 37°C for 2 h. Samples were reconstituted with 120 µl EIA buffer (for a 12-fold dilution), vortexed and held at 4°C until assayed. Extraction efficiency was measured by comparing mean recovery of extracted (n=5) versus non-extracted (n=5) 2000 cpm tritiated CORT (H^3). Mean extraction efficiency of H^3 was 101% ± 2% s.d. (n=9 extractions) with an overall CV of 1.64%. Extracted plasma samples (50 µl) were analysed in duplicate using commercial enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI, note: kit validation for first-time use in tuatara is detailed below) containing 96-well plates coated with rabbit polyclonal anti-sheep IgG antibody. CORT-specific acetylcholinesterase (AChE) tracer and sheep CORT antiserum were added to sample wells and placed on an orbital shaker for 2 h. Plates were washed five times, developed for 1 h on an orbital shaker and subsequently read at 405 nm. The concentration of CORT was calculated by comparing results to a standard curve. Samples that did not yield CORT concentrations within the 10 – 90% bound range were re-assayed after an appropriate dilution.

To calculate the intra-assay co-efficient of variation (CV), three quality-control (QC) samples with binding levels of 20%, 50% and 65% were prepared in extracted

tuatara plasma and analysed (in duplicate) repeatedly throughout one assay. Replicate aliquots (n=9) of the QCs yielded similar plasma CORT concentrations (ng/ml) at each level, with %CVs of 8.3, 10.4 and 13.9 for the 20%, 50% and 65% bound QCs, respectively. The mean intra-assay CV for all QCs was 10.9%. Inter-assay CVs were calculated from the same QCs placed at the start and end wells (in duplicate) of each assay plate (n=8). Mean inter-assay CVs were 11.4% (20% bound QC), 12.2% (50% bound QC), and 13.5% (65% bound QC). The mean inter-assay CV for all QCs was 12.4%. Serial dilutions at 100%, 80%, 60%, 40%, 20%, and 10% of pooled tuatara plasma samples showed good parallelism to the standard curve for CORT over the assay standard range. Multiple linear regression analysis was used to provide confirmation that the serial dilutions were comparable to the CORT standard curve provided (P=0.746). Linear regression equations were: $\%B/B_0$ = 48.9 – 42.3 logcort (r^2=99.2, P<0.001) for the serial dilution and: $\%B/B_0$ = 5.6 – 40.7 logcort for the standard curve (r^2=99.1, P<0.001). To determine the minimum detectable concentration (MDC) of CORT I assayed B_0 wells (n=16, each in duplicate) on a single plate and calculated mean CORT concentration minus two standard deviations. Results showed a MDC of 0.03 ng/ml, which corresponds with the MDC supplied by the EIA kit manufacturers.

2.3.5 Statistical analyses

Data analyses were carried out using R v3.0.0 statistical software (R Development Core 2008) and Prism 6 (Graphpad Software Inc.). All data were checked for normal distributions and homoscedasticity, and if necessary, were transformed to meet assumptions for parametric statistical tests. A linear mixed effects regression (LMER) model was fitted using the 'lme4' (Bates, 2013) package in R to investigate the influence of sex, reproductive condition, T_b, body condition and duration of capture-restraint (0 – 24 h) on the CORT response. Log-transformed CORT was the response variable, predictors were group (male, gravid female, non-gravid female), duration of capture-restraint (h), T_b and body condition were fixed main effects (with significant interaction terms included), and tuatara ID was included as a random effect to account for repeat sampling of individuals for baseline CORT and CORT response values. The 'languageR' package (Baayen, 2011) was used to compute P-values based on Markov-chain Monte Carlo (MCMC) sampling.

Significance for all tests was assumed at $p < 0.05$. To quantify the magnitude of the mean CORT response (ng/ml) over the 24 h capture-restraint period, I calculated the area under the curve (AUC) in Prism 6 using the trapezoid rule, where AUC values reflect an 'integrated CORT response' for each group (Cockrem and Silverin, 2002; Narayan *et al.*, 2011a). Baseline values were set at zero and negative values were not included. I used linear models (LMs) and post-hoc contrast tests to compare body condition between groups, and to investigate the relationship between T_b and baseline CORT secretion in all groups. I used a LMER model to compare T_b between groups and sample times (at baseline and CORT response sampling), with tuatara ID included as a random effect to account for repeat T_b sampling of individuals.

2.4 Results

2.4.1 CORT response to 24 h capture-restraint

I analysed the effect of acute 24 h capture-restraint on CORT secretion profiles in gravid, non-gravid and male tuatara by fitting a linear mixed effects regression (LMER) model to CORT concentrations from 0 to 24 hours. Neither T_b nor body condition were significant predictors of the CORT response across groups and were therefore excluded from the final model (Table 2.1). Mean baseline CORT concentrations (ng/ml) were significantly higher in gravid females (5.75 ± 1.21) compared to non-gravid females (1.05 ± 0.15) and males (1.30 ± 0.19) (Fig 2.1a, Table 2.1). The CORT response over 24 h was significantly dampened in gravid females compared with non-gravid females and males (Fig 2.1b-d, Table 2.1). By determining AUCs, I was able to quantify the magnitude of the mean CORT response (ng/ml) over a 24 h period of capture-restraint stress (Fig 2.1b-d). Gravid females had the lowest mean integrated CORT response (ng/ml/24 h) at 2.61, followed by males at 11.13 (4-fold greater than gravid females) and non-gravid females at 13.36 (5-fold greater than gravid females). Peak secretion of CORT occurred at 1.5 hours for gravid females, and at 12 hours for both non-gravid females and males (Fig 2.1b-d).

Table 2.1: Results from linear mixed effects regression models explaining variation in measures of baseline and stress response corticosterone secretion (ng/ml) as a function of time (0-24 h) after explaining variation accounted for by the fixed effects of sex, reproductive condition, duration of capture-restraint and the interaction of these effects[1].

Fixed effect predictor	Est.	Lower 95%CI	Upper 95%CI	*P*-MCMC
gravid females (Intercept)	0.792	0.673	0.910	<0.001
non-gravid females	-0.626	-0.788	-0.462	<0.001
males	-0.656	-0.825	-0.495	<0.001
capture-restraint (gravid females)	-0.002	-0.014	0.010	0.718
capture-restraint × non-gravid females	0.017	0.001	0.035	0.041
capture-restraint × males	0.023	0.007	0.041	0.011

[1] Coefficient estimates (positive or negative) are shown and indicate direction of the linear regression from the specified intercept. 95% credible intervals (CI) are shown. *P*-values based on MCMC sampling are shown.

2.4.2 Relationships with body temperature and body condition

Neither T_b (P>0.05) nor body condition (P>0.05) were significant predictors for the CORT response. I found a significant positive relationship between T_b and baseline CORT concentrations (ng/ml) in gravid females only (LM, logCORT = -0.603 + 0.108 T_b, r^2=0.210, P=0.015, Fig 2.2). T_b was comparable across groups at baseline ($F_{2,86}$=1.147, P=0.322) and during CORT response ($F_{2,86}$=0.128, P=0.880) sampling. An increase in T_b from baseline to CORT response sampling occurred in all groups (LMER, P<0.001). Mean T_b (°C) at baseline sampling was 12.2 ± 0.4 for gravid females, 11.7 ± 0.3 for non-gravid females and 11.4 ± 0.4 for males, and at CORT response sampling was 13.8 ± 0.3 for gravid females, 13.9 ± 0.2 for non-gravid females and 13.7 ± 0.2 for males. On average, males were larger in body size, but I did not find a significant difference in body condition scores across groups ($F_{2,86}$=1.374, P=0.259).

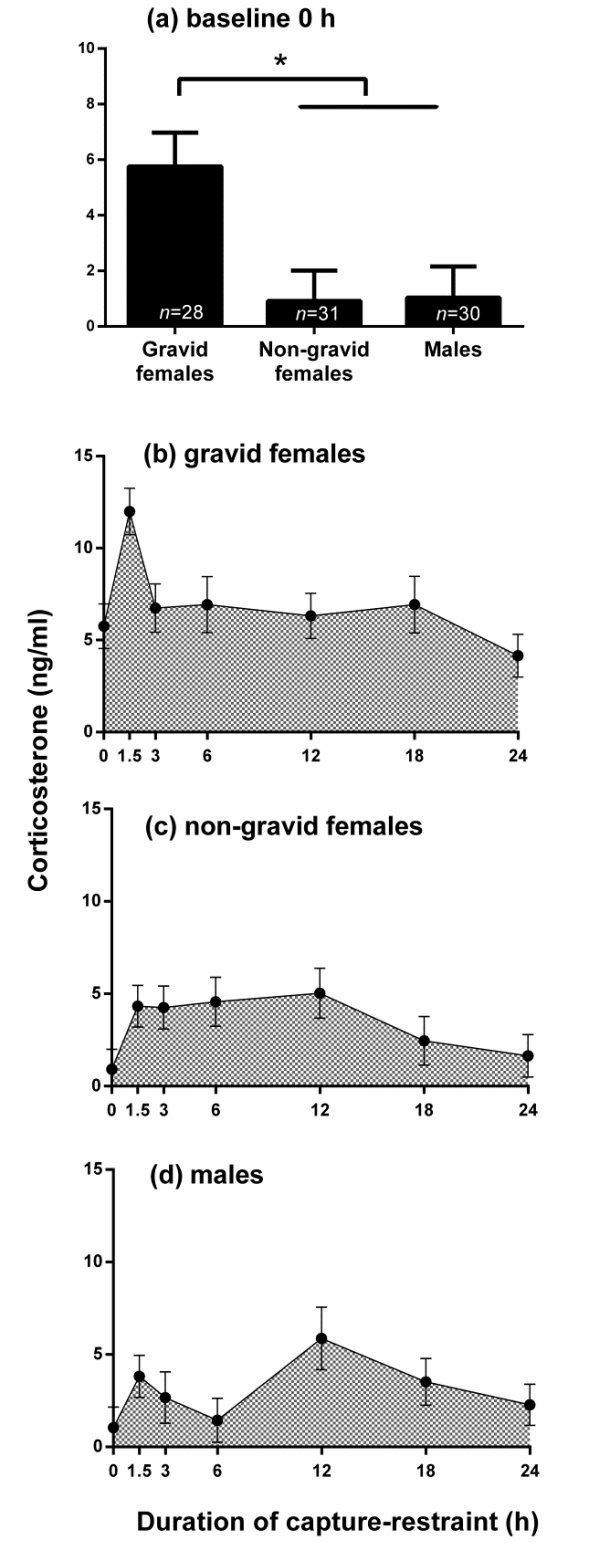

Figure 2.1: Patterns in (a) baseline CORT concentration (ng/ml) and the 24 h CORT response in (b) gravid female, (c) non-gravid female and (d) male tuatara (*Sphenodon punctatus)* over a period of 24 h capture-restraint. Data points represent means ± SE. Baseline CORT concentrations are also indicated by dashed lines in (b)-(d). Shaded curves delineate the integrated CORT response (ng/ml/24 h) for each group calculated by area under the curve (AUC).

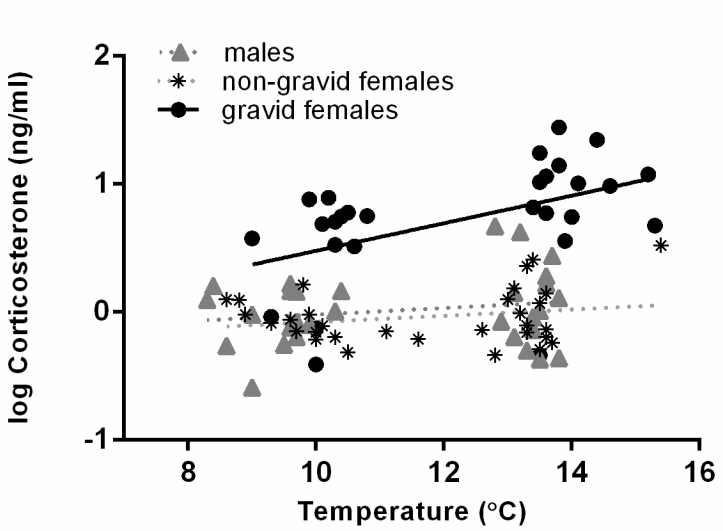

Figure 2.2: Relationship between cloacal body temperature (°C) and the log of baseline CORT concentration (ng/ml) in male (grey dotted line w/triangles), non-gravid female (black dotted line w/ stars) and gravid female (black solid line w/circles) tuatara (*Sphenodon punctatus).* A significant relationship was observed in gravid females only (r^2=0.210, *P*=0.015).

2.5 Discussion

As expected, gravid females had significantly higher baseline CORT concentrations compared with non-gravid females (and males) during the nesting life-history stage. This result is consistent with a previous study on wild tuatara, in which the mean plasma concentration of baseline CORT in gravid females was almost twice that of non-gravid females and males (Tyrrell and Cree, 1998). Here, I established that the CORT response is dampened (4- to 5-fold) in gravid females, compared with non-gravid females and males during 24 h capture-restraint. A dampened CORT response has been observed in other oviparous reptiles during the gravid life-history stage, including female tree lizards (Woodley and Moore, 2002) green turtles (Jessop *et al.*, 2000) and olive ridley turtles (*Lepidochelys olivacea*) (Valverde *et al.*, 1999). This naturally leads to the question of why some gravid oviparous reptile species modulate the CORT response (specifically down-regulating CORT secretion) during a physiologically and behaviourally challenging situation (Jessop, 2001; Moore and Jessop, 2003). Possible explanations for this trait include: adaptive

interruption of certain stress response reactions (e.g. flight response) to nest successfully, adaptive maternal effects and/or programming, lack of awareness towards certain stressors, or an inability to mount a further CORT response. Furthermore, alternative physiological mechanisms such as changes in binding proteins (CBGs) and/or CORT receptors at target tissues may play a role in the CORT response to stress in gravid individuals (Romero, 2002).

Gravid reptiles that are actively nesting could have a suppressed CORT response to interrupt or shield activation of the emergency life-history stage (in which self-maintenance takes precedence) in order to successfully carry out nesting (Wingfield *et al.*, 1998). Suppression of the CORT response during nesting has been observed in other amniotes, including reptiles, and may serve to increase reproductive success and overall fitness. Female sea turtles show a dampened CORT response during nesting activities, and maintain suppression of a CORT response in the face of predation attacks (Jessop *et al.*, 2004a), thermal stress (Jessop *et al.*, 2000) and overcrowding (Jessop and Hamann, 2004) in order to successfully nest. Similarly, studies in birds report modulation of the CORT response in order to increase reproductive success (Lendvai *et al.*, 2007; Wingfield *et al.*, 1994; Wingfield and Sapolsky, 2003), with a few authors noting increased CORT secretion that leads to nest abandonment (Spee *et al.*, 2010; Strasser and Heath, 2013; Thierry *et al.*, 2013).

An attenuated CORT response could also be a product of adaptive maternal effects such as protecting eggs/embryos from potential deleterious effects of hormones, or allowing maternal programming of offspring to occur. Maternal hormones have been discovered in yolk of numerous vertebrate species, including birds (Almasi *et al.*, 2012; Hayward and Wingfield, 2004), fish (Manire *et al.*, 2004) and reptiles (Rhen *et al.*, 2006). Increased levels of maternal CORT can affect exposure of developing embryos to CORT (Cree *et al.*, 2003; Uller *et al.*, 2009), which can influence phenotype, behaviour, and fitness performance measures. For example, maternal sources of CORT can influence offspring size and sex-ratio (Uller *et al.*, 2009; Warner *et al.*, 2009). Pregnant females with increased plasma CORT concentrations produced offspring that exhibited decreased anti-predator behaviour in garter snakes (*Thamnophis elegans*) (Robert *et al.*, 2009) and decreased activity,

sprint speed, and motivation to run in common lizard hatchlings (*Lacerta vivipara*) (Belliure *et al.*, 2004; Meylan and Clobert, 2004). Future studies could investigate and possibly manipulate maternal transfer of CORT to eggs of tuatara to further explore the potential relationship with yolk hormones and subsequent offspring development, phenotype and performance.

It is also possible that gravid females that lack a stress response to capture-restraint (or in which the stress response is attenuated) do not actually perceive the stressor as a threat during specific life-history events such as nesting, oviposition, or parturition. Therefore, behavioural resistance to stressors could effectively modulate CORT secretion to maintain reproductive behaviour (Jessop *et al.*, 2004a; Jessop *et al.*, 2000; Jessop *et al.*, 1999b). Threat perception could be tested in tuatara by intensifying or compounding the stressor (e.g. heat/cold-stress or exposure to predators in addition to capture-restraint stress) to see if there is a behavioural threshold that activates the CORT response. Finally, the absence of a significant CORT response in gravid females could be attributed to the fact that gravid females are operating at maximal CORT secretion during nesting or are experiencing a form of chronic stress, and are simply unable to further mount a response. This could be tested further by administering an ACTH challenge (Cartledge and Jones, 2007; Preest *et al.*, 2005) (addressed in Appendix A).

The functional significance of elevated baseline CORT in gravid oviparous reptiles (and the potential relationship with oviposition) has received little attention, even though several oviparous reptile species exhibit elevated concentrations of baseline CORT concentrations directly preceding oviposition (Cree and Tyrrell, 2001; Jessop, 2001; Moore and Jessop, 2003). Female tuatara have a unique egg development/maintenance strategy, retaining their eggs for the longest known period in reptiles (7-9 months) (Cree *et al.*, 1992). Therefore, elevated baseline CORT concentrations during the nesting life-history stage in tuatara cannot be attributed to sexual receptivity or ovulation, as these mating events are greatly separated in time from nesting. Prior studies on tuatara (Cree and Tyrrell, 2001) and marine iguanas (Rubenstein and Wikelski, 2005) show that baseline CORT concentrations decline shortly after oviposition, which suggests a possible role in

the timing of egg-laying, and other studies suggest hormonal control of ovulation and parturition in viviparous and oviparous reptiles (Jones and Guillette, 1982). For example, it was shown that embryonic production of CORT may trigger parturition in viviparous southern snow skinks (*Niveoscincus microlepidotus*) (Girling and Jones, 2006), and exogenous elevation of baseline CORT concentrations in gravid eastern three-lined skinks (*Bassiana duperreyi*) induced 'premature' oviposition (Radder *et al.*, 2008). These findings suggest that up-regulating baseline CORT secretion during nesting could function to stimulate oviposition in gravid reptiles. This could be investigated further by experimentally manipulating baseline CORT secretion in gravid females to see if there is a relationship between CORT secretion and oviposition (Appendix A).

In the current study, neither T_b nor body condition were significant predictors of the CORT response in tuatara. However, I found a significant positive relationship between T_b and baseline CORT secretion in gravid females only, with no relationship observed between T_b and the CORT response in non-gravid females or males. An increase in T_b occurred between capture and CORT response sampling; however, this is most likely due to sheltered conditions (in the capture bags), rather than a physiological response (Tyrrell and Cree, 1998). As individuals in this study were captured and sampled over two nights, T_b varied over a limited range (8°C -15°C) and approached the lower end of field T_b observed in tuatara (emergence and activity occurs between ~6°C and 25°C (Besson and Cree, 2010; Saint Girons *et al.*, 1980)); therefore, it would be useful to investigate CORT secretion at increased T_b over a wider range (see Chapter Three). Likewise, I did not find a relationship between body condition and baseline CORT secretion or the CORT response in females (gravid and non-gravid) and males. The influence of body condition on CORT secretion is quite variable among reptile species, with some studies reporting a significant influence (Baker *et al.*, 2013; Dayger *et al.*, 2013; Dunlap and Wingfield, 1995; Hews and Baniki, 2013; Jessop *et al.*, 2004c; Waye and Mason, 2008), no relationship (Baker *et al.*, 2013; Jessop *et al.*, 2004c; Selman *et al.*, 2012) or both (Wikelski and Romero, 2003). Interestingly, Wilkelski and Romero (2003) found that CORT secretion is elevated in marine iguanas (*Amblyrhynchus cristatus*) that have body condition scores below a critical threshold level, but they did not find a

significant correlation between CORT and body condition in individuals with scores above the threshold level. Studies incorporating an expanded range of body condition scores (including populations in obvious poor condition) could clarify the relationship between body condition and CORT secretion in tuatara, and may identify a critical threshold level (related to body condition) at which CORT secretion is affected. Nonetheless, the influence of T_b and body condition (and possible physiological thresholds of both measurements) on CORT secretion are areas of research that require further study in reptiles.

2.6 Conclusions

I found that the CORT response is dampened in gravid female tuatara during the nesting life-history stage, which suggests modulation of CORT secretion to support nesting success. Perhaps down-regulating CORT secretion during exposure to acute stressors facilitates reproductive success by ensuring that nesting activities carry on regardless and that eggs/embryos are potentially shielded from elevated levels of maternal CORT. I confirmed that female reproductive condition is significantly correlated with baseline CORT secretion, with gravid females showing levels that are significantly higher than non-gravid females (and also males). The functional significance of elevated baseline CORT secretion in gravid females during nesting is unclear; therefore, it would be useful to carry out experimental studies involving hormone manipulation (in which CORT secretion is increased or decreased) in gravid females (see Appendix A). This would provide insight into whether CORT is being secreted at maximal levels during nesting, if/how it plays a role in oviposition and nesting behaviour, and how CORT modulation contributes to reproductive success and overall fitness in tuatara, the sole extant representative of an ancient order of reptiles.

CHAPTER 3

Body temperature is correlated with the corticosterone stress response in tuatara (*Sphenodon punctatus*)[1]

3.1 Abstract

When vertebrates experience environmental, physiological or social challenges, the glucocorticoid (GC) stress response is activated and rapid secretion of GCs ensues as an essential life process to promote survival. Ambient temperature affects essential life processes in most organisms. In reptiles, ambient temperature directly influences internal body temperature (T_b), which has downstream effects on physiology and behaviour. In a natural setting, fluctuations in temperature occur routinely, yet despite its relevance to essential processes in reptiles, T_b has received limited attention with regards to its influence on the GC stress response. In this study, I examined the influence of T_b on baseline secretion of corticosterone (CORT), the main GC in reptiles, and on the CORT response to capture-restraint across a range of T_b in free-living tuatara (*Sphenodon punctatus*) (males, non-gravid females and gravid females) I also tested the effect of an acute increase in T_b on the magnitude of the CORT response. I confirmed a positive correlation between T_b and the CORT response to capture in all groups, and a positive correlation between T_b and baseline CORT in gravid females only. Furthermore, gravid females only exhibited a significant CORT response to capture at high T_b , which in combination with results from Chapter Two, suggests a response threshold at ~22-25°C (i.e. at or

[1] This chapter is based on the following manuscript submitted to Physiological and Biochemical Zoology (*in review*) with minor modifications: Anderson L, Nelson N, Cree A. Increased body temperature amplifies the corticosterone stress response in tuatara (*Sphenodon punctatus*).

above selected temperature). Overall, this study confirms experimentally that T_b is correlated with CORT secretion in a cold-adapted reptile and should be considered in studies on other ectotherms, especially when fluctuations in T_b are experienced.

3.2 Introduction

Free-living organisms are regularly exposed to environmental fluctuations. To cope with daily, seasonal and annual variation in environmental conditions, individuals adjust their physiology and behaviour to maintain essential processes and optimise fitness. One way that vertebrates adjust to environmental conditions is through secretion of glucocorticoids (GCs) from adrenal tissues. GC secretion upholds everyday maintenance such as energy intake and cell provisioning of glucose (Landys *et al.*, 2006), and variation in GC secretion (ranging from hourly to annually) is observed within and between species. Furthermore, when individuals experience environmental perturbations, the hypothalamo-pituitary-adrenal (HPA) axis is activated and GC secretion is rapidly increased to promote immediate survival (Boonstra, 2013; Busch and Hayward, 2009; Cockrem, 2013). Although numerous studies have investigated factors that affect GC secretion (both baseline and in response to stressors) (Baker *et al.*, 2013; Boonstra, 2013; Breuner *et al.*, 2008; Creel *et al.*, 2013; Wingfield, 2013), few studies consider the influence of body temperature (T_b).

Ambient temperature affects essential life processes in most organisms. In ectotherms, most notably reptiles, ambient environmental temperature directly influences T_b, which has downstream effects on several aspects of physiology and behaviour (Adams *et al.*, 1989; Firth *et al.*, 1989; Jasnic *et al.*, 2013; Seebacher *et al.*, 1999). Despite its relevance to physiological processes in reptiles, T_b has received limited consideration in the scientific literature with regards to GC secretion. In reptiles, corticosterone (CORT) is the primary GC in reptiles, and as in other vertebrates, an adrenocortical stress response is observed (Cockrem, 2013; Romero, 2002). Numerous intrinsic and extrinsic factors are associated with CORT secretion in reptiles. Examples include sex, reproductive condition, body condition, disease, population, season and pathogens (Baker *et al.*, 2013; Barry *et al.*, 2010; Berger *et al.*,

2005; Eikenaar *et al.*, 2012; Selman *et al.*, 2012). However, the relationship between T_b and baseline CORT secretion in reptiles remains unclear. Some studies suggest a relationship while others question it, and the direction and magnitude of the relationship (if any) varies within and between species (Baker *et al.*, 2013; Cree *et al.*, 2003; Dupoue *et al.*, 2013; Li *et al.*, 2011; Sykes and Klukowski, 2009; Tyrrell and Cree, 1998). Among the limited studies that exist, most have focussed on the influence of T_b on baseline CORT secretion. To my knowledge, only two studies have experimentally tested the influence of T_b on the CORT response in reptiles. Furthermore, the species used in these studies were both warm-adapted snakes that were exposed to a either a sub-optimal temperature treatment in captivity (Dupoue *et al.*, 2013) or to 1 h acute temperature treatments (semi-aquatic snakes were warmed or cooled within physiological limits) (Sykes and Klukowski, 2009). These studies do not clarify the effect of T_b on the CORT response in free-living terrestrial ectotherms. Moreover, neither study considers the association between T_b and CORT secretion (baseline CORT and the CORT response) in gravid females.

Tuatara (*Sphenodon punctatus*) are a cool-climate terrestrial reptile with a low mean preferred body temperature (T_{sel}) of about 21°C in laboratory conditions (Besson and Cree, 2010), although T_b in basking individuals (in both laboratory and field conditions) can reach 25-30°C (Barwick, 1982; Besson and Cree, 2010; Cree, 2014; Saint-Girons *et al.*, 1981; Stebbins, 1958). Hence, tuatara provide a valuable opportunity to examine the influence of T_b (at capture in natural conditions and in warmer increments up to T_{sel}) on CORT secretion in a cold-adapted ectotherm. Here, I conduct an observational field study to examine baseline CORT secretion and the CORT response across a range of ambient environmental temperatures, and experimentally test whether elevated T_b during capture-restraint stress affects the CORT response.

Tuatara are protected reptile species endemic to New Zealand and natural populations are currently restricted to isolated offshore islands (Jones and Cree, 2012). They are the only extant representatives of the order Rhynchocephalia, the sister group to squamates (Jones, 2008). Tuatara have a seasonally distinct reproductive cycle with mating taking place in the austral autumn (February –

March) and nesting in the austral spring (October – November). Individual females do not reproduce annually; instead, ovulation and nesting events occur only every 2-5 years (Cree *et al.*, 1992; Moore *et al.*, 2009a; Refsnider *et al.*, 2010). Therefore, males and non-gravid females can be found in the same population at the same time (along with gravid females in spring), which allows for control of sex, reproductive condition, population and season.

Baseline CORT secretion in tuatara is relatively low and variation is observed seasonally, but not diurnally (Tyrrell and Cree, 1998), and a CORT response to capture-restraint stress is detected (Tyrrell, 1993; Tyrrell and Cree, 1998; Chapter Two). A previous study has reported observations of a positive relationship between T_b and baseline CORT secretion in males (but not females) during summer and winter (Tyrrell and Cree, 1998). More recently, a positive relationship between T_b and baseline CORT secretion was observed in gravid females (but not males or females) during spring, whereas no association was observed between T_b and the CORT response (Chapter Two); however, the range of T_b (8 -15°C) observed in that study was narrow and at the lower end of T_b observed in natural populations of tuatara.

There were two main aims to the present study. First, I examined whether T_b is associated with baseline CORT secretion and the magnitude of the CORT response in natural populations of tuatara. Second, I experimentally tested whether an acute increase in temperature influenced the magnitude of the CORT response in males, non-gravid females and gravid females. I predicted that the CORT response would be amplified at increased temperatures compared to the control temperature treatment (T_b at capture) in all groups. At the same time, I examined whether gravid females are capable of mounting a significant CORT response during nesting when simultaneously exposed to capture-restraint and an acute temperature increase. A significant CORT response in gravid females would suggest that the ability to secrete CORT is not fundamentally impaired during the nesting life-history stage, but is seasonally modulated.

3.3 Materials and methods

3.3.1 Influence of natural variation in body temperature on CORT secretion

3.3.1.1 Study sites and animals

Wild adult tuatara (*Sphenodon punctatus*) were captured and sampled from Stephens Island/Takapourewa (40° 40' S, 174° 00' E), North Brother Island (41° 6' S, 174° 25' E), Lady Alice Island (35° 53' S, 174° 43' E) and Taranga Island ('35° 58' S, 174° 43' E) in New Zealand during the austral spring (November) and austral autumn (March) in the 2011/2012 and 2012/2013 seasons. Emerged individuals were captured by hand at night between 19:00 h and 03:00 h and were subsequently assigned to an appropriate group based on sex and reproductive status (male, non-gravid female, gravid female). Tuatara reach sexual maturity at approximately 13 years of age (~ SVL ≥ 170mm), and previous studies have distinguished males and females by examining secondary sex characteristics such as head and body size/shape and spine/crest morphology (Cree *et al.*, 1991a; Dawbin, 1982). Gravid females (present in spring only) were selectively captured at or near a nesting rookery on Stephens Island/North Brother Island and reproductive status was inferred through abdominal palpation for shelled eggs (Mitchell *et al.*, 2006; Refsnider *et al.*, 2013). Locations of nesting rookeries on Lady Alice Island and Taranga Islands are not known; therefore, for the purpose of analyses, all females captured during the spring season on these two islands were classified as non-gravid adults (although a proportion of sampled females could have been gravid). I intentionally include data from four island sites in order to expand the range of T_b observed, as samples collected within a site often showed limited variation in T_b.

3.3.1.2 Study design

Male (n=72), non-gravid female (n=77) and gravid females (n=30) were sampled over a two year study period to examine the influence of T_b on baseline CORT secretion and the CORT response across a wide range of environmental temperatures. Blood samples were collected within 10 min of capture to determine baseline CORT secretion (described in section 4.1.3) and tuatara were subsequently

subject to 3 h of capture-restraint stress in cloth bags at ambient environmental temperatures. Upon completion of capture-restraint, all individuals were re-bled to determine the CORT response.

3.3.1.3 Blood sampling protocol

To determine baseline CORT concentrations, a blood sample (up to 1 ml) was collected within 10 min of capture from the base of the tail with a heparinized 23-gauge needle and 1 ml syringe. In previous studies of tuatara, no significant effect of bleeding effort (time from capture to blood sample collection) was observed on baseline CORT concentrations in blood samples collected within 20 min of capture (Chapter Two; Tyrell, 1993). After baseline samples were taken, individuals underwent 3 h capture-restraint whereupon a second blood sample (up to 1 ml) was taken. Depending on field conditions, blood samples were separated either by centrifuge (5 min at 2000 rpm) or under normal gravity for 6 to 8 h at 4°C (Reimers *et al.*, 1983; Sheriff *et al.*, 2011). Plasma was transferred into cryogenic vials with a micropipette, stored in a cryogenic dry shipper (Thermo Fisher Scientific™, Arctic Express™ Dual 10, Waltham, Massachusetts, USA) or in a freezer at -20°C until return to the laboratory, and then stored at -80°C until assayed.

3.3.1.4 Internal body temperature (T_b) and body condition measurements

Internal body temperature (T_b) was recorded with a cloacal thermocouple (Fluke® Multimeter, model: 179, Everett, Washington, USA, reported accuracy ±1°C) prior to taking blood samples (both baseline and CORT response) for each individual. Morphometric measurements were taken after capture-restraint and CORT response blood sampling was completed. Individual animals were weighed with a spring scale (Pesola AG, Barr, Switzerland) to determine mass (g), and snout-vent length (mm), tail length (mm), and tail regeneration length (mm) were measured with a ruler. I calculated tail-corrected mass (TCM) following Newman *et al.* (1994) to account for tail-loss and regeneration. Body condition scores for all individuals were obtained by generating standardized residuals from a regression of logTCM vs.

log SVL for each group (males, non-gravid females, gravid females) (Schulte-Hostedde *et al.*, 2005).

3.3.2 Effect of acute increased body temperature on the CORT response

3.3.2.1 Study site and animals

Wild adult tuatara were captured and sampled on Stephens Island (40° 40' S, 174° 00' E) in Cook Strait, New Zealand over 3 nights during the 2012 spring season (21-24 October). Emerged individuals were caught by hand at night between 19:00 h and 03:00 h and were subsequently grouped by sex and reproductive status (male, non-gravid female, gravid female). Gravid females were selectively captured at a nesting rookery and reproductive status was inferred through abdominal palpation for shelled eggs.

3.3.2.2 Study design

Male (n=14), non-gravid female (n=14) and gravid female (n=14) tuatara were subject to an acute increase in temperature to test whether the CORT response is amplified at higher temperatures. Blood samples were collected within 10 min of capture to determine baseline CORT secretion (described in section 3.3.1.3). Next, individuals were randomly assigned to an air temperature treatment of either 'control' (ambient), 'warm' (20°C) or 'hot' (25°C). Individuals were subject to 3 h of capture-restraint stress in perforated cardboard postal tubes (10 cm x 50 cm) at assigned temperature treatments and upon completion, were re-bled to determine the effect of capture-restraint and temperature treatment on CORT secretion.

Internal T_b was taken immediately prior to each blood sample and body condition was determined for each individual (described in section 4.1.4) after the second blood sample was taken. Temperature treatments of 20°C and 25°C were chosen to approximate the average ('warm' treatment) and highest ('hot' treatment) T_b, respectively, measured in the parallel observational study. These temperatures are physiologically appropriate (i.e. not considered lethal or expected to invoke signs of obvious 'heat-stress' such as panting) and fall within the range of T_b observed in

(and selected by) individual tuatara in field and laboratory settings (Barwick, 1982; Besson and Cree, 2010; Corkery, 2012; Cree, 2014; Saint Girons, 1980; Werner and Whitaker, 1978). Treatment conditions were achieved by elevating air temperatures in a quiet confined space in the research house with convection heaters. Labelled postal tubes housing individuals were placed uniformly at an even height (established by a pilot study to determine optimal height for each temperature treatment), and air temperatures (within the grouping of postal tubes at each treatment height) for both treatments were monitored every 15-30 min with a thermocouple to ensure consistency of treatments. Individuals assigned to control treatments (ambient air temperature) were housed in postal tubes outside the research house.

3.3.3 Determination of plasma CORT concentrations

Plasma CORT concentrations for all samples were determined as described in Chapter Two. Briefly, I randomly selected plasma samples (29/plate) for assay that were thawed at room temperature and spun in a centrifuge at 5000 rpm for 5 min at 4°C to remove any residual debris. I added 600 µl of freshly distilled dichloromethane to extract 10 µl aliquots of plasma samples. Samples were incubated 15 min at room temperature, snap-frozen in a -70°C ethanol bath, decanted into glass tubes and then dried in a vacuum oven at 37°C for 2 h. Samples were reconstituted with 120 µl enzyme immunoassay (EIA) buffer (for a 12-fold dilution), vortexed and held at 4°C until assayed. I determined extraction efficiency by comparing mean recovery of extracted (n=5) versus non-extracted (n=5) 2000 cpm tritiated CORT (H^3). Mean extraction efficiency of H^3 was 106% ± 2% s.d. (n=16 extractions) with an overall CV of 2%.

Extracted plasma samples (50 µl) were analysed in duplicate using commercial enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI, USA) containing 96-well plates coated with rabbit polyclonal anti-sheep IgG antibody. CORT-specific acetylcholinesterase (AChE) tracer and sheep CORT antiserum were added to sample wells, and the plate was placed on an orbital shaker for 2 h. Plates were washed five times with wash buffer solution, developed for 1 h on an orbital shaker and subsequently read at 405 nm. The concentration of CORT was calculated by

comparing results to a standard curve. Samples that did not yield CORT concentrations within the 10 – 90% bound range were re-assayed after an appropriate dilution. The mean intra-assay CV was 10.9% and the mean inter-assay CV was 15.4%.

3.3.4 Statistical analyses

Data analyses were carried out using R v3.2.0 statistical software (R Development Core 2013) and Prism 6 (Graphpad Software Inc.). Residual plots were checked for normal distributions and homoscedasticity, and if necessary, data were transformed to meet assumptions for parametric statistical tests. Linear mixed effects regression (LMER) models were fitted using the 'lme4' package (Bates, 2013) in R to analyse 1) the relationship between T_b and CORT secretion across a range of ambient environmental temperatures, and 2) the effect of increased T_b on the CORT response in males, non-gravid females and gravid females subject to 3 h of capture-restraint stress in controlled temperature treatments. Log-transformed CORT was the response variable in all LMER models. Final model selection was determined by likelihood ratio tests comparing the addition of random and fixed effects to intercept-only baseline models.

In analysis 1 (influence of natural variation in T_b on CORT secretion), input variables included fixed main effects of time (0 h, 3 h), T_b, body condition, and all interaction terms. Random effects of year and tuatara ID (nested within site) were included to account for annual variation, repeat sampling of individuals for baseline CORT and CORT response values, and random unmeasured variation between sites. Final models were fitted to data from each group for spring (males, non-gravid females, gravid females) and autumn (males, non-gravid females).

In analysis 2 (effect of increased T_b on the CORT response), input variables included fixed main effects of time (0 h, 3 h), body condition and temperature treatment (ambient, warm, hot), with all interaction terms included. A random effect of tuatara ID was included in both models to account for repeat sampling. Due to small sample size, I pooled data for males and non-gravid females for the experimental analyses, as sex was not a significant predictor in this study (P>0.05); therefore final models

were fitted to data from non-gravid adults (males, non-gravid females) and gravid adults (gravid females). Body condition did not have a significant influence on CORT secretion for any group (P>0.05) and was not included in final models. I used linear models (LMs) and post-hoc contrast tests to compare T_b and body condition between groups within each controlled temperature treatment at 0 h and 3 h sample times, and I used a LMER model to compare change in T_b from 0 h to 3 h in each temperature treatment (with tuatara ID included as a random effect to account for repeat sampling). The 'lmerTest' package (Kuznetsova, 2013) was used to compute P-values for final models and significance was assumed at P< 0.05.

3.4 Results

3.4.1 Influence of natural variation in body temperature on CORT secretion

Internal T_b ranged from 8°C to 20°C in spring and 12°C to 23°C in autumn (Fig. 3.1). Body temperature at capture (0 h) and at 3 h after capture was not significantly different among groups (males, non-gravid females, gravid females) in either season (LM, P>0.05). However, T_b was significantly increased at 3 h after capture (LMER, P<0.001), which is most likely due to sheltered conditions (in capture bags) (Chapter Two; Tyrrell and Cree, 1998).

As expected, I observed a significant CORT response to capture restraint in males and non-gravid females in both seasons (Fig. 3.1, Table 3.1), and observed a dampened CORT response in gravid females in spring (Fig. 3.1, Table 3.1a). I found that T_b was not correlated with baseline CORT in males and non-gravid females during spring or autumn (Table 3.1); however, T_b is positively correlated with CORT secretion in gravid females in spring (Table 3.1a, Fig. 3.1a). Furthermore, a higher CORT response was observed at higher T_b in all groups in each season (including gravid females - present in spring only) (Fig. 3.1b, d, Table 3.1).

Body condition scores were not significantly different between seasons (spring, autumn) or among groups (male, non-gravid female, gravid female) (LM, $F_{4, 355}$=0.309, P=0.872). Body condition was not correlated with baseline CORT for any

group/season (LMER, P>0.05). However, in spring only, a significant positive correlation was observed between body condition and the CORT response in non-gravid females (LMER, P<0.001) and in gravid females (LMER, P=0.035), with a weak trend observed in males (LMER, P=0.09). No significant interactions between T_b and body condition were observed for any group/season (LMER, P>0.05, Table 3.1).

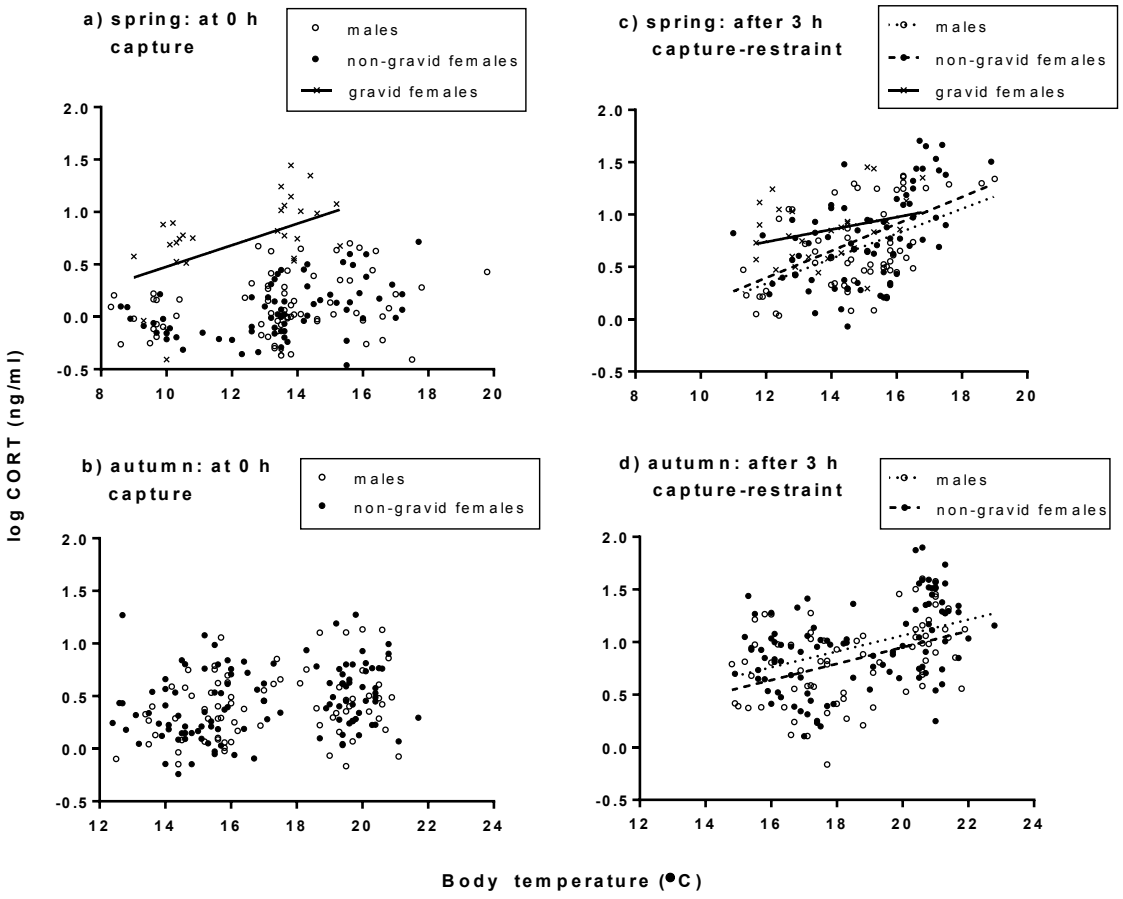

Figure 3.1: Relationships between internal body temperature (°C) and log CORT concentration (ng/ml) in tuatara (*Sphenodon punctatus*). Data are shown for male (open circles, dotted lines), non-gravid female (black circles, dashed lines) and gravid female (crosses, solid lines) tuatara at 0 h after capture in a) spring and b) autumn and after 3 h capture-restraint in c) spring and d) autumn. Data points represent each individual sampled and regression lines show positive relationships. Note: gravid females are only present during spring.

Table 3.1: Results from LMER models explaining variation in baseline CORT and the CORT response in tuatara (*S. punctatus*) accounting for fixed effects of time (0 h capture and 3 h capture-restraint), internal body temperature, body condition and the interaction of these effects (if applicable). Models were fitted to data for males, non-gravid females, and gravid females in spring (a), and for males and non-gravid females in autumn (b). [1]Gravid adults are present in spring season only.

Fixed effect predictor	estimate	s.e.	*T* value	*P* value
a) Spring				
males				
0 h (Intercept)	0.198	0.162	1.215	0.378
3 h	0.571	0.063	9.027	<0.001*
T_b	-0.029	0.199	-1.428	0.156
body condition	-0.028	0.052	-0.527	0.599
3 h x body condition	0.112	0.066	1.691	0.093·
3 h x T_b	0.116	0.028	4.049	<0.001*
T_b x body condition	0.021	0.019	1.115	0.267
3 h x T_b x body condition	-0.004	0.030	-0.134	0.893
Non-gravid females				
0 h (Intercept)	0.349	0.273	1.279	0.367
3 h	0.631	0.049	12.867	<0.001*
T_b	-0.004	0.017	-0.291	0.771
body condition	-0.046	0.040	-1.146	0.254
3 h x body condition	0.210	0.059	3.552	<0.001*
3 h x T_b	0.084	0.021	3.937	<0.001*
T_b x body condition	-0.013	0.022	-0.593	0.554
3 h x T_b x body condition	-0.023	0.035	-0.680	0.498
gravid females[1]				
0 h (Intercept)	0.780	0.064	12.074	<0.001
3 h	- 0.027	0.070	-0.383	0.704
T_b	0.109	0.026	4.143	<0.001*
body condition	0.121	0.054	2.233	0.035*
b) Autumn				
Males				
0 h (Intercept)	0.430	0.144	2.967	0.171
3 h	0.349	0.055	6.246	<0.001*
T_b	0.018	0.017	1.056	0.334
3 h x T_b	0.055	0.021	2.561	0.012*
Non-gravid females				
0 h (Intercept)	0.396	0.168	2.354	0.086
3 h	0.545	0.050	10.791	<0.001*
T_b	-0.017	0.019	-0.855	0.378
3 h x T_b	0.031	0.018	1.740	0.084·

3.4.2 Effect of acute increased body temperature on the CORT response

Body temperature at capture (0 h) was comparable between groups assigned to all temperature treatments (LM, $P>0.05$). As expected, significantly higher T_b was reached at 3 h in the 'warm' and 'hot' temperature treatments, compared to the ambient 'control' treatment (LMER, $P<0.05$, Fig. 3.2a).

The increase in T_b was not significantly different among groups (males, non-gravid females, gravid females) within each temperature treatment at 3 h (LM, $P>0.05$). Mean T_b after 3 h reached 12.6 ± 0.7°C in the 'control' treatment, 17.3 ± 0.4°C in the 'warm' treatment and 21.4 ± 0.4°C (which is T_{sel} for tuatara) in the 'hot' temperature treatment (Fig. 3.2a). Body condition was comparable among groups (LM, $P>0.05$), and was not a significant predictor of CORT secretion in this experiment (LMER, $P>0.05$).

Males and non-gravid females (pooled data) exhibited a significant CORT response in all temperature treatments (Fig. 3.2b, Table 3.2a). The magnitude of the CORT response was not significantly different between the 'control' and 'warm' treatments (LMER, $P>0.05$); however, a positive trend in the 'hot' temperature treatment was observed and suggests an amplified CORT response in this treatment, compared to 'control' and 'warm' treatments (LMER, $P=0.09$) (Fig. 3.2b, Table 3.2a). Gravid females maintained a dampened CORT response in the 'control' and 'warm' temperature treatments (LMER, $P>0.05$), but exhibited a significant CORT response in the 'hot' temperature treatment (LMER, $P=0.04$) (Fig. 3.2c, Table 3.2b).

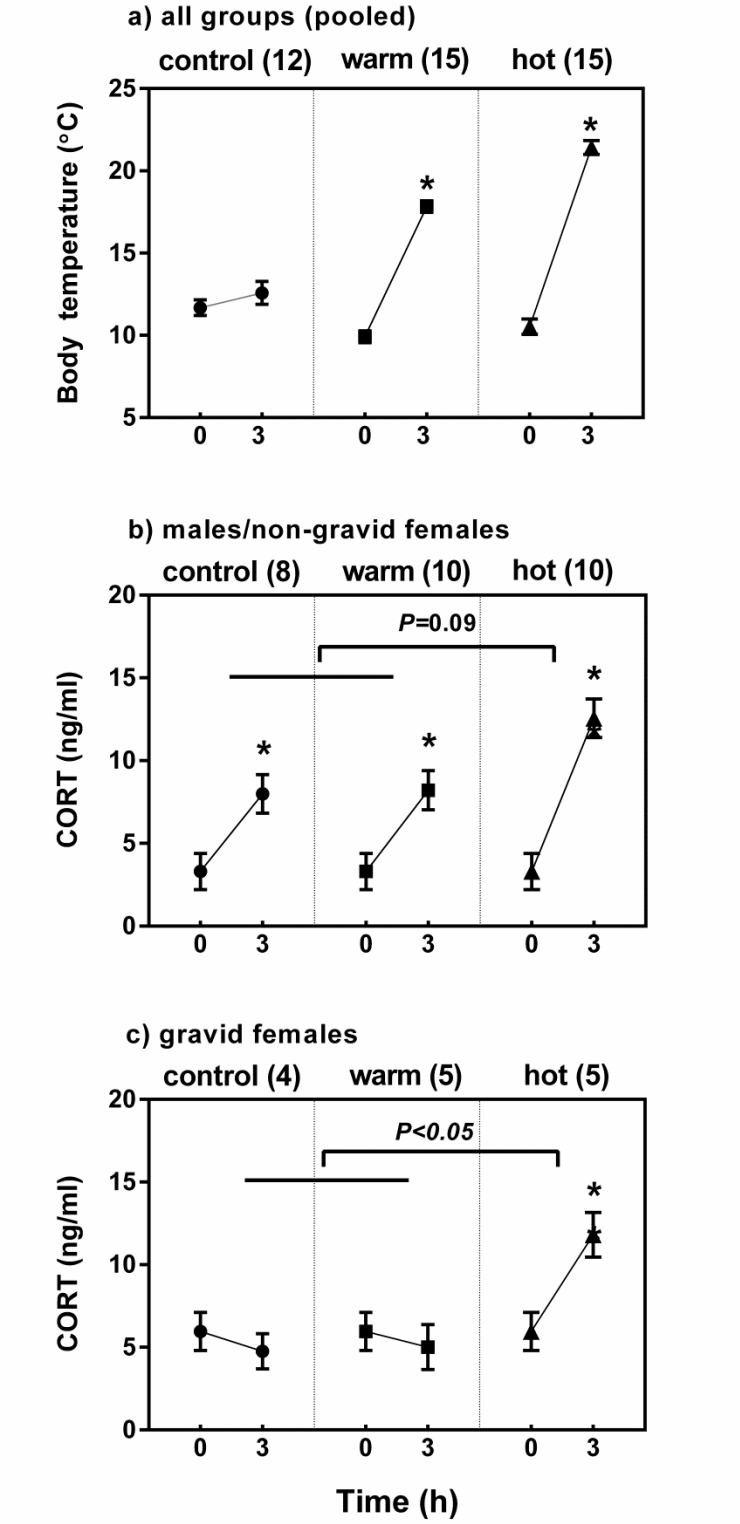

Figure 3.2: Effect of acute increased body temperature on the CORT response in tuatara (*Sphenodon punctatus*). Data show change from 0 h to 3 h in a) internal body temperature, CORT secretion in b) male and non-gravid female and c) gravid female tuatara subject to capture-restraint and temperature treatments of 'control' (mean T_b = 12.6°C), 'warm' (mean T_b = 17.3°C) and 'hot' (mean T_b = 21.4°C). Data points represent mean ± s.e. Asterisks indicate a significant change observed after 3 h compared to baseline (0 h), and bars above treatments show the difference in magnitude of the CORT response. Numbers in brackets indicate sample size.

Table 3.2: Results from LMER models explaining variation in measures of baseline CORT and the CORT response in tuatara (*S.punctatus*) accounting for fixed effects of time , temperature treatment (control, warm, hot) and the interaction of these effects for a) male/non-gravid female tuatara and b) gravid female tuatara.

Fixed effect predictor	estimate	s.e.	*T* value	*P* value
a) males/non-gravid females				
0 h control (Intercept)	0.560	0.673	0.910	<0.001
0 h warm	-0.084	0.101	-0.831	0.410
0 h hot	-0.027	0.105	-0.253	0.801
3 h x control	0.343	0.097	3.575	<0.001*
3 h × warm	0.096	0.126	0.076	0.454
3 h × hot	0.223	0.132	1.694	0.094·
b) gravid females				
0 h control (Intercept)	0.823	0.121	6.818	<0.001
0 h warm	-0.217	0.171	-1.275	0.218
0 h hot	0.034	0.155	0.219	0.828
3 h x control	-0.145	0.124	-1.166	0.268
3 h × warm	0.096	0.126	0.076	0.454
3 h × hot	0.360	0.161	2.243	0.046*

3.5 Discussion

I examined the relationship between natural variation in ambient temperature and baseline CORT and the CORT response in tuatara, and experimentally tested whether acute increased body temperature affects the magnitude of the CORT response. Here, I confirm for a cold-adapted reptile a significant correlation between body temperature and CORT secretion. My results reveal three interesting patterns: (i) a significant positive correlation between T_b and baseline CORT secretion in gravid females only, (ii) a significant positive correlation between T_b and the CORT response in males and females (gravid and non-gravid), and (iii) an acute increase in T_b approaching a threshold at/near ambient temperatures of 25°C elicits a significant CORT response in gravid females (which at lower temperatures fail to show a response), and may influence the magnitude of the CORT response in

males and non-gravid females (though the latter is a marginal trend). These findings are important as many behavioural and physiological processes in ectotherms are sensitive to changes in ambient environmental temperature (Bonnet *et al.*, 2013; Cartland and Grimmond, 1994; Finkler, 2006; Gaby *et al.*, 2011; Lawrence, 1997; Lourdais *et al.*, 2008), and should therefore be considered in physiological studies utilising ectotherms as model organisms.

3.5.1 Body temperature is correlated with baseline CORT in gravid females only

I observed a positive correlation between T_b and baseline CORT in gravid females only, and found no significant correlation between T_b and baseline CORT in males or non-gravid females. These results agree with findings from a previous study investigating patterns of CORT in male, non-gravid female and gravid female tuatara (Chapter Two). Other studies have reported inconsistent effects of T_b on baseline CORT in reptiles, with reports of a positive relationship, negative relationship or no relationship at all (Baker *et al.*, 2013; Romero and Wikelski, 2006). Tyrrell and Cree (1998) found a positive correlation between T_b and baseline CORT in male but not female tuatara, during summer and winter months, which the authors suggested could be a product of increased T_b promoting specific behavioural activity that coincides with increased baseline CORT. This hypothesis could potentially explain the elevated baseline CORT observed in gravid females in the current study, i.e. it is possible that higher T_b in gravid females may facilitate (or be connected with) migration to nesting grounds, nest digging and/or oviposition activities in the spring nesting season. Cree and Tyrrell (2001) associated nesting behaviour (digging/nest guarding) with elevated baseline CORT in gravid female tuatara; however T_b was not measured/reported in that study. Therefore, further research is required to better understand the relationship and connection between T_b, behavioural activities, and baseline CORT.

3.5.2 The CORT response is higher at warmer ambient temperatures

The magnitude of the CORT response was significantly higher in tuatara sampled at warmer ambient temperatures, with effectively higher T_b. Numerous studies have

reported increased rates for physiological processes at warmer temperatures in reptiles, including hormone secretion (Callard *et al.*, 1975; Narayan and Hero, 2014), blood circulation (Dunlap, 2006; Maclean *et al.*, 1975), gestation (Michel *et al.*, 2013), and metabolic processes such as growth, digestion and locomotion (Aidam *et al.*, 2013; Bonnet *et al.*, 2013; Cartland and Grimmond, 1994; Gillooly *et al.*, 2001; Narayan *et al.*, 2012; Polo-Cavia *et al.*, 2012; Preest and Cree, 2008; Tamplin *et al.*, 2013).

In addition, I found that body condition is positively correlated with the CORT response in the spring season, but not in the autumn season. In tuatara, it is possible that a stronger CORT response may contribute to better body condition in the spring. Food resources are reduced during the winter period, with a seasonal shift of increased prey availability occurring in late spring/summer (Walls, 1981); therefore, the presence of a stronger CORT response may reflect increased foraging activity in certain individuals leading to better body condition. Many studies have found a positive correlation between CORT secretion and feeding behaviour (reviewed in Landys et al., 2006), and experimentally elevated CORT levels have been shown to stimulate foraging activities (Breuner and Hahn, 2003; Crossin *et al.*, 2012; Lancaster *et al.*, 2008). Therefore, it may be beneficial to have a stronger CORT response during specific seasons, environmental conditions and/or life-history events. Alternatively, my results may indicate that individuals in better body condition are able to mount a stronger CORT response compared to individuals with reduced body condition. This prediction could be examined by looking at the CORT response across a range of body conditions, seasons and environmental scenarios (see Chapter Four). The role of CORT in regulating foraging activity could be examined experimentally in a controlled setting by applying exogenous CORT and monitoring feeding behaviour. Results from such studies may clarify the relationship between body condition and the magnitude of the CORT response.

3.5.3 Acute increased body temperature elicits a CORT response in gravid females

In my experimental study, I confirmed that an acute increase in T_b can elicit a CORT response in gravid females. I did not observe a significant CORT response in gravid

females in both 'control' and 'warm' temperature treatments; yet a significant CORT response was exhibited by gravid females in the 'hot' temperature treatment. My experiment demonstrates, as previously suggested (Chapter Two), that gravid females are not operating at maximal CORT secretion at capture (despite their high baseline CORT levels) and are indeed able to mount a significant CORT response during the nesting life-history stage. In addition, I also observed a weak trend between an acute increase in T_b and the CORT response in males and non-gravid females in the 'hot' temperature treatment, though the trend status is marginal. I recognise that my sample sizes are small (particularly for gravid females); therefore it would be useful to conduct a larger-scale experiment in which T_b extends slightly above T_{sel} to confirm and extend this result.

Nevertheless, the significant findings observed in gravid females in my experimental study, paired with the weak trend observed in males and non-gravid females, provides compelling evidence that exposure to acute temperature increases (up to and most likely above T_{sel}) can increase the magnitude of the CORT response in a cold-adapted reptile. This may be explained by an increase in the rate of hormone secretion at higher temperatures (as discussed in section 3.5.2), or possibly an effect of exposure to compound stressors (capture-restraint plus T_b increase/inability to thermoregulate). In a natural setting, ectotherms have the capacity to buffer changes in ambient air temperatures (and thus actively thermoregulate T_b) through behavioural actions such as seeking shade, retreating to burrows, or basking (Besson and Cree, 2010; Kearney et al., 2009; Seebacher et al., 1999); therefore, exposure to increased temperatures (without the option to regulate T_b) may explain my findings.

In a similar study to mine, the Children's python (*Antaresia childreni*) exhibited an higher CORT response when subject to a cold temperature treatment of 17°C (which is well below the pythons' T_{sel} of ~29°C). The authors suggest a response threshold exists to allow individuals to cope with suboptimal temperatures when unable to thermoregulate. It would be interesting to see if the same response was observed at higher T_b (above T_{sel}) in this species. Likewise, the magnitude of the CORT response to suboptimal temperatures could be tested in tuatara by decreasing T_b. However, as

tuatara are a cold-adapted reptile and have been observed at ambient temperatures as low as 5-6°C , with a critical thermal minimum temperature of 0.7°C (Besson and Cree, 2011), it would be likely that a 'cool' temperature for tuatara could possibly approach 0°C and there could be a risk of tissue/cell freezing (Gillooly *et al.*, 2001). In a recent study on the Qinghai toad-headed lizard (*Phrynocephalus vlangalii*) (a cold-adapted alpine reptile with ambient daytime T_b ranging from 0 – 20°C), CORT secretion was examined after exposure to acute cold-temperature treatments (at 0.1-0.5°C) of varying durations. No significant response to temperature treatments was observed; however, the lizards used in this study were wild-caught, held in captivity for two weeks before the experiment and neither baseline CORT secretion values, the effect of captivity nor inter-individual fights were considered, which may have contributed to the observed results (Li *et al.*, 2011). Similarly, the influence of both 'cold' and 'warm' temperatures on the CORT response in water snakes (*Nerodia sipedon*) was investigated (in which acute change from T_b at capture was ± 10°C), and no effect of acute temperature on the magnitude of the CORT response was observed (Sykes and Klukowski, 2009). Water snakes are known to regularly experience acute temperature change (i.e. moving from basking to swimming) and are able to function over a wide range of T_b (~7-30°C), which may explain the lack of response to temperature in this species. Clearly, more research investigating the interplay between temperature, thermoregulatory capacity, and CORT secretion in ectotherms is needed.

Increased CORT secretion supports immediate survival during situations where challenges are numerous or perceived as more intense/stressful (Bonnet *et al.*, 2013; Graham *et al.*, 2012). I recommend the examination of nesting behaviour (digging, oviposition, guarding etc.) of gravid female tuatara to test the prediction that they will abandon (or possibly will not initiate) nesting activities at a specific temperature threshold (at or above T_{sel}) at which a significant CORT response is produced. Studies in green turtles have reported that gravid females exposed to heat-stress or severe shark attacks maintain a dampened CORT response and continue nesting (Jessop *et al.*, 2004a; Jessop, 2001).

3.6 Conclusions

I confirm a significant correlation between T_b and CORT secretion in a cold-adapted ectotherm. The magnitude of the CORT response is positively correlated with ambient temperatures in male and female (gravid and non-gravid) tuatara, whereas baseline CORT is positively correlated with ambient temperatures in gravid females only. An acute increase in T_b to T_{sel} (~21°C T_b) elicits a significant CORT response in gravid female tuatara, and may increase the CORT response in males and non-gravid females (although this is a weak trend). Possible explanations for this observation include a CORT response threshold at T_{sel}, an increase in the rate of hormone secretion caused by higher temperatures, or an effect of compound stressors (capture-restraint plus T_b increase /inability to thermoregulate). These findings are important as many behavioural and physiological processes in ectotherms are sensitive to changes in ambient temperature and should therefore be considered in physiological studies utilising ectotherms as model organisms, especially where variation in ambient temperatures is experienced. Furthermore, these results will inform conservation efforts involving capture-restraint (which constrains normal thermoregulatory behaviour), such as in population monitoring, research programmes, translocation and captive management programmes.

CHAPTER 4

The corticosterone stress response varies among island populations of tuatara (*Sphenodon punctatus*) and is associated with linear ecological attributes[1]

4.1 Abstract

The most widely used indicators of physiological stress in free-living vertebrates are glucocorticoid (GC) hormones. Measurement of GC concentrations in body fluids or products to assess population status and/or to monitor conservation efforts is central to stress physiology research. However, in order to fully understand the stress physiology of a species, and to make comparisons among populations, it is essential to consider seasonal patterns in GC secretion and incorporate ecological variation. I compared baseline corticosterone (CORT), the primary GC in reptiles, and the 3 h CORT response to capture-restraint among four populations of a rare rhynchocephalian reptile, the tuatara (*Sphenodon punctatus*) during the non-breeding and breeding seasons to determine inter-population variation in CORT secretion. Then, I used principal components analysis (PCA) to simplify five ecological attributes into two component axes that explained 96% of the ecological variance among populations, and used principal component regression to test linear ecological predictors (PC1, PC2) of CORT secretion. Finally, I determined plasma testosterone (T) in a subset of two populations as a proxy for reproductive activity

[1] This chapter is based on the following manuscript submitted to Animal Conservation (*in review*) with minor modifications: Anderson L, Nelson N, Towns D, Cree A. The corticosterone stress response varies among island populations of tuatara (*Sphenodon punctatus*) and is predicted by linear ecological attributes.

and examined associations between T and CORT secretion. I found that baseline CORT secretion was similar among populations during the non-breeding and breeding seasons; however, the CORT response to capture-restraint varied significantly among populations. In general, baseline CORT increased, and the CORT response decreased, from the non-breeding season to the breeding season. I found significant correlations between the CORT response and habitat factors of latitude, tuatara density and seabird abundance (PC1 axis) and demogenetic factors of sex ratio and genetic diversity (PC2 axis); however, no correlations were observed between baseline CORT along both axes. Testosterone secretion was not associated with CORT secretion. Nonetheless, inter-population variation in T was observed in males (but not females) and was positively linked with increased baseline CORT from the non-breeding season to the breeding season, suggesting that male reproductive activity may drive the seasonal increase in baseline CORT.

4.2 Introduction

Glucocorticoid hormones (corticosterone (CORT) or cortisol) are the most widely used indicators of physiological stress in free-living vertebrates (Wikelski and Cooke, 2006). The use of CORT in body fluids (plasma) or products (urine, faeces) as a rapid tool to assess population health and/or to monitor conservation efforts is instrumental in stress physiology research (Busch and Hayward, 2009; Dantzer *et al.*, 2014; Wikelski and Cooke, 2006). When individuals experience unpredictable challenges (i.e. stressors) that affect internal balance or homeostasis, a complex physiological response cascades through the hypothalamo-pituitary-adrenal (HPA) axis to stimulate CORT secretion (as either a gradual increase in baseline CORT or as an immediate CORT stress-response) to help individuals cope with stressors and to promote overall fitness and survival (Wingfield and Kitaysky, 2002; Wingfield *et al.*, 1998). Even so, patterns of CORT secretion are not universally consistent; variation is observed among species, and within and among populations (Cockrem, 2013; Creel *et al.*, 2013; Love *et al.*, 2013).

Variation in CORT secretion can be linked to intrinsic factors (sex, reproductive status, age) and to environmental, social and/or physiological stressors such as habitat modification (either natural or anthropogenic causes), as well as seasonal changes in food availability, temperature and/or weather, and in reaction to increased energy demands required during reproduction, territorial disputes and migration (Baker *et al.*, 2013; Berner *et al.*, 2013; Hamann *et al.*, 2007; Holding *et al.*, 2014b; Owen *et al.*, 2014; Rubenstein and Wikelski, 2005; Taylor *et al.*, 2014; Woodley *et al.*, 2003).

It is generally agreed that baseline CORT meets energetic demands associated with predictable life-history stages, whereas the CORT response is a reaction to unpredictable and/or challenging events (Tokarz and Summers, 2011; Wingfield *et al.*, 1998). Modulation of CORT secretion helps individuals cope with their specific environment to effectively increase fitness (Moore and Jessop, 2003; Romero, 2002). Baseline CORT is commonly elevated during the breeding season to support energetic requirements associated with reproductive activities, and similarly, individuals experiencing reduced breeding opportunities or living in harsh environments have been found to dampen their CORT response during the breeding season, which may serve to increase chances of reproductive success (Ebensperger *et al.*, 2013; Holding *et al.*, 2014b; Jessop *et al.*, 2000; Moore and Jessop, 2003; Romero, 2006; Wingfield *et al.*, 1998; Wingfield and Sapolsky, 2003).

Examining patterns of CORT secretion within a population can be a useful tool to assess and monitor overall health, and may be a way to identify unpredicted challenges and gauge an individual's ability to respond to stressors (Bonier *et al.*, 2009; Breuner *et al.*, 2008). That said, gaining a good understanding of factors that influence CORT secretion, while considering environmental context, is important. Many studies have examined factors influencing CORT secretion (both baseline CORT and the CORT response); however, studies that incorporate intrinsic, seasonal, and ecological/anthropogenic attributes to assess sources of variation in CORT secretion among wild populations are limited (Boonstra, 2013; Dantzer *et al.*, 2014).

Here, I compare patterns in the secretion of CORT, the main GC in reptiles, among four wild populations of tuatara (*Sphenodon punctatus*) during the non-breeding and breeding seasons, and examine potential attributes that may explain variation in CORT secretion. Natural populations of tuatara are currently restricted to isolated offshore islands (Jones and Cree, 2012) with considerable variation in ecological attributes such as island size, sex ratio, body condition, climate, conspecific density, seabird abundance, food availability, human activity, history of introduced Pacific rats (*Rattus exulans*), genetic diversity and demography (Cree, 2014; Gaze, 2001). Tuatara are an ideal species to examine relationships between CORT secretion and influential factors (intrinsic, seasonal, ecological/anthropogenic) due to the variety of environmental conditions observed among island populations and to their distinctive breeding cycle.

Tuatara have seasonally distinct reproductive activity with breeding (mating and ovulation) occurring in autumn (February – March) and nesting in spring (October – December), though not all females ovulate and nest each year (Cree, 2014). Male reproductive activity peaks in late summer/early autumn (January-March) (Saint Girons and Newman, 1987) and includes prenuptial displays, courtship, mating, and territorial defence (Gans *et al.*, 1984; Moore *et al.*, 2009b). Male reproductive success is highly skewed, with only 25-30% of individuals successful in securing a mate (Moore *et al.*, 2009a). Spermatogenesis and plasma testosterone concentrations in males follow an annual cycle, being low during the winter, rising in the spring, and peaking in midsummer to early autumn during the mating period (Cree, 2014). Female tuatara exhibit an extended reproductive cycle, carrying eggs in the oviduct for 6–8 months and producing one clutch of eggs every 2-9 years, depending on the population (Cree *et al.*, 1992; Moore *et al.*, 2009a; Refsnider *et al.*, 2010). Female tuatara show highest levels of testosterone and estradiol when in pre-ovulatory condition during the breeding season, however; only ~10 - 50% of females in a given population are ovulating (hence receptive to a mate) during the breeding season (Cree, 2014; Mitchell *et al.*, 2010; Moore *et al.*, 2009a; Tyrrell *et al.*, 2000).

Baseline CORT in tuatara is generally low (Cree and Tyrrell, 2001; Tyrrell and Cree, 1998) and a significant CORT response is observed to capture-restraint (Chapter Two; Tyrrell and Cree, 1998). Studies examining variation in CORT secretion among tuatara populations are limited, with only one previous study comparing CORT secretion between two northern populations (a rat-free population vs a rat-inhabited population), in which presence of rats was associated with an elevation in the 3 h and 18 h CORT response but did not influence baseline CORT (Tyrrell *et al.*, 2000).

Seasonal variation in baseline CORT has been observed in the population on Stephens Island, where baseline CORT was highest in November for females and February/May for males (Tyrrell and Cree, 1998); however, seasonal patterns of baseline CORT secretion (in other island populations) and seasonal patterns of the CORT response have not been investigated. I found a positive correlation between body temperature (T_b) and the CORT response in tuatara (Chapter Three), and that body condition is not correlated with baseline CORT or the CORT response (Chapter Two; Chapter Three). Here, I make further use of samples collected in Chapter Three (along with new samples) to examine inter-island variation of CORT secretion in two seasons, while effectively controlling for (and examining) the influence of temperature and body condition.

In this study, I examined variation in CORT secretion (baseline CORT and the CORT response to capture-restraint) among four populations during the non-breeding and breeding seasons. I predicted that (i) baseline CORT would vary among populations during each season due to contrasting environmental attributes; (ii) baseline CORT would be higher during the breeding season (compared to the non-breeding season) in all populations, as tuatara exhibit energetically demanding reproductive behaviours/activity during the breeding season (Moore *et al.*, 2009b; Saint Girons and Newman, 1987); and, (iii) the CORT response would be higher in northern populations (compared to Cook Strait populations), in both seasons, due to a milder climate (e.g. warmer T_b) and past/recent rat presence. I tested relevant ecological predictors of CORT secretion (through principal component analysis) to identify significant factors that may contribute to variation among populations. Secondly, I

examined the association between reproductive activity (as indicated by testosterone (T) concentrations) and modulation of CORT secretion from the non-breeding season to the breeding season in a subset of two populations with contrasting seasonal modulation of baseline CORT.

4.3 Material and methods

4.3.1 Study sites and animals

Wild adult tuatara (*Sphenodon punctatus*) were captured and sampled from four island populations in New Zealand during the non-breeding (austral spring) and breeding (austral autumn) seasons. Samples were collected from Stephens Island/Takapourewa (40° 40' S, 174° 00' E), North Brother Island (41° 6' S, 174° 25' E), Lady Alice Island (35° 53' S, 174° 43' E) and Taranga Island ('35° 58' S, 174° 43' E) during the non-breeding season (November 2011) and breeding season (March 2011* and March 2012, *Lady Alice and Taranga Islands only). Both Lady Alice Island and Taranga Island CORT concentrations were not significantly different between the 2011 and 2012 breeding seasons (LMER, $P>0.05$), therefore I pooled 2011/2012 breeding season samples for each site to increase sample size for analyses.

Samples were obtained from adult males and adult non-gravid females during each season. Tuatara reach sexual maturity at approximately 13 years of age (~ SVL ≥ 170mm) (Castanet *et al.*, 1988), at which point sex can be identified by examining head shape, spine shape, and body size/shape (Cree, 2014; Cree *et al.*, 1991a; Dawbin, 1982). Gravid females with eggs are only present during the non-breeding season and are difficult to locate on lower density islands (pers. obs.); therefore I chose not to include gravid females in my sampling regime.

4.3.2 Sampling protocol

Samples were collected at night (between 20:00 h and 04:00 h) from hand-captured individuals that had emerged from their underground burrows. To determine baseline CORT concentrations, a blood sample (up to 1 ml) was collected within 10

min of capture from the base of the tail with a heparinized 23-gauge needle and 1 ml syringe. In previous studies of tuatara, no significant effect of bleeding effort (time from capture to blood sample collection) was observed on baseline CORT concentrations in blood samples collected within 20 min of capture (Chapter Two; Tyrrell, 1993). After baseline samples were taken, individuals underwent 3 h capture-restraint in cloth capture bags either outdoors or in a research house (depending on the field site), whereupon a second blood sample (up to 1 ml) was taken.

Internal body temperature (T_b) was recorded with a cloacal thermocouple (Fluke® Multimeter, model: 179, reported accuracy ±1°C) prior to taking blood samples (both baseline CORT and CORT response) for each individual, as T_b has been shown to influence CORT secretion in tuatara (Chapter Three). After the 3 h blood sampling was completed, individuals were weighed with a 1000 g spring scale (Pesola AG, Switzerland) to determine mass (g), and snout-vent length (SVL) (mm), tail length (mm), and tail regeneration length (mm) were measured with a ruler to determine body condition. Depending on field conditions, blood samples were separated either by centrifuge (5 min at 2000 rpm) or under normal gravity for 6 to 8 h at 4°C (Reimers *et al.*, 1983; Sheriff *et al.*, 2011). Plasma was transferred into cryogenic vials with a micropipette, stored in a cryogenic dry shipper at -190°C (Thermo Scientific™, Arctic Express™ Dual 10) or in a freezer at -20°C until return to the laboratory, and then stored at -80°C until assayed.

4.3.3 Linear ecological predictors: Principal components analysis (PCA) of ecological attributes

I used principal components analysis (PCA) to identify combinations of ecological attributes that explain variation between island populations. A correlation matrix was used to generate PCA scores from ecological attributes (Table 4.1). Principal component axes with eigenvalues ≥1.0 were retained and a minimum absolute value of ≥ 0.4 for loading coefficients was chosen as a requirement for significance of an ecological variable in each PCA axis (Field *et al.*, 2012).

Table 4.1: Description and ecological attributes of study populations

Population	*Latitude*	*Island size (ha)*	*Tuatara density (#/ha)*	*Relative seabird density*	*History of introduced rats*	*Genetic Diversity (H)*	*Sex ratio (M:F)*	*Female gravidity rate (%/yr)*	*References*
Stephens	40° 40' S	150	674	high	None	0.6	1 : 1	21.9	(Cree, 2014; Gaze, 2001; Hay *et al.*, 2010; Moore *et al.*, 2010; Newman, 1987; Newman *et al.*, 1994)
North Brother	41° 6' S	4	134 - 159	high	None	0.1	3.2 : 1	10.8	(Cree, 2014; Cree *et al.*, 1991a; Gaston and Scofield, 1995; Gaze, 2001; Grayson *et al.*, 2014; Hay *et al.*, 2010; Mitchell *et al.*, 2010; Nelson *et al.*, 2002; Wilson, 2010)
Lady Alice	35° 53' S	155	13.6 – 17.7	med	Rats eradicated in 1994	0.7	1 : 1	28.8 -32	(Cassey and Ussher, 1999; Cree, 2014; Cree *et al.*, 1995; Gaze, 2001; Hay *et al.*, 2010; Newman *et al.*, 1994; Towns *et al.*, 2007; Tyrrell *et al.*, 2000)
Taranga	35° 58' S	500	1.1	low	Rats eradicated in 2011	0.7	1 : 1.5	27	(Buxton *et al.*, 2013; Cree, 2014; Cree *et al.*, 1995; Gaze, 2001; Hay *et al.*, 2010)

4.3.4 Reproductive activity: Testosterone (T) secretion

I determined plasma testosterone (T) concentrations (at both 0 h and 3 h) in a subset of plasma samples obtained in this study from the Stephens (males: *n*=10, females: *n*=20) and North Brother (males: *n*=15, females: *n*=15) populations in Cook Strait. Plasma T concentrations can be used as a gauge for reproductive activity in males, where increased T implies increased sperm production, territoriality, courtship, and mating events (Cree *et al.*, 1992; Gillingham *et al.*, 1995). In females, plasma T concentrations are normally lower than males; however, results can be used to gauge percentage of reproductively active females in each population, as elevated T concentrations (obtained within 3 h of capture) in February–March distinguish female tuatara in ovulatory condition (Cree *et al.*, 1991b).

4.3.5 Enzyme-Immunoassay

4.3.5.1 Corticosterone

Plasma CORT concentrations for all samples were determined as described in Chapter Two. Mean extraction efficiency of tritiated CORT (H^3) was 106% ± 5% s.d. (*n*=25 extractions) with an overall CV of 5.18%. The mean intra-assay CV was 9.9% and the mean inter-assay CV was 15.4%.

4.3.5.2 Testosterone

Plasma samples from the Cook Strait populations (Stephens and North Brother Island - NBI) obtained during the March 2012 breeding season (*n*=120) were sorted into male and female samples (30/plate) and extracted for assay as described for CORT in Chapter Two. Serial dilutions at 100%, 80%, 60%, 40%, 20%, 10%, 5% and 1% of pooled male tuatara plasma samples showed good parallelism to the standard curve for testosterone (T) over the assay standard range (P>0.05). Samples were reconstituted with 2 ml EIA buffer (for a 200-fold dilution), vortexed and held at 4°C until assayed.

Extraction efficiency was measured by comparing mean recovery of extracted (*n*=5) versus non-extracted (*n*=5) 2000 cpm tritiated T [1,2,6,7-H^3]. Extracted plasma samples (50 µl) were analysed in duplicate using commercial enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI) containing 96-well plates coated with mouse monoclonal anti-rabbit IgG antibody. Testosterone-specific acetylcholinesterase (AChE) tracer and sheep T antiserum were added to sample wells and placed on an orbital shaker for 2 h. Plates were washed five times, developed for 1 h on an orbital shaker and subsequently read at 412 nm. The concentration of T was calculated by comparing results to a standard curve; all samples yielded T concentrations within the 20 – 80% bound range. Mean extraction efficiency of [1,2,6,7-H^3] was 100% ± 5% s.d. (*n*=4 extractions) with an overall CV of 4.9%. The mean intra-assay CV was 10.9% and the mean inter-assay CV was 12.4%.

4.3.6 Statistical analyses

Data analyses were carried out using R v3.2.0 statistical software (R Development Core 2013) and Prism 6 (Graphpad Software Inc.). All data were checked for assumptions of normality and were transformed if necessary. To generate body condition scores, I calculated tail-corrected mass (TCM) following Newman *et al.* (1994) to account for tail-loss and regeneration, pooled data across both seasons (separated by sex) then generated standardized residuals from a regression of logTCM vs. logSVL (Schulte-Hostedde *et al.*, 2005).

Linear models (LM) and linear mixed effects regression models (LMER) were fitted using the 'lme4' package (Bates, 2013) to analyse: 1) inter-population variation in CORT secretion, body condition and T secretion, and 2) linear ecological predictors (PCA axes) of CORT secretion. Final models were selected through forward selection by comparing the addition of fixed and random effects to intercept-only baseline models (Field *et al.*, 2012), whereupon parameter estimates were obtained with the summary() function in R. The 'lmerTest' package (Kuznetsova, 2013) was used to compute *P*-values for parameter estimates and statistical significance was assumed at $P < 0.05$.

First, I determined variation in CORT secretion and body condition among populations during the non-breeding and breeding seasons. Sex was not a significant predictor in any of the final models (P>0.05); therefore sexes were pooled for all analyses. Final models for CORT secretion included response variables of log transformed baseline CORT and the CORT response (3 h CORT – 0 h CORT) and input variables of season (non-breeding, breeding), population (Stephens, NBI, LA, Taranga), linear covariates of internal body temperature (T_b) and body condition, and an interaction term of season* population. The final model for body condition included a response variable of body condition score and input variables of season (non-breeding, breeding), population (Stephens, NBI, LA, Taranga) and an interaction term of season* population.

Second, I examined linear ecological predictors (PCA axes) of CORT secretion through principle component regression models. Sex was not a significant predictor in any of the final models (P>0.05); therefore sexes were pooled for all analyses. The final models included a response variable of log transformed CORT response (3 h CORT – 0 h CORT) and input variables of season (non-breeding, breeding), linear covariates of PCA Axes (PC1 and PC2) and an interaction terms of season * PCA axes (PC1 and PC2).

Lastly, I determined variation in T secretion between a subset of two populations and examined associations with CORT secretion. The final model for T secretion included a response variable of T (ng/ml) and input variables of hour (0 h, 3 h), population (Stephens, NBI), sex (M, F) and an interaction term of population * sex. The final model for CORT and T association included a response variable of log transformed CORT and input variables of hour (0 h, 3 h), population (Stephens, NBI), sex (M, F) and a linear covariate of T. A random effect of tuatara ID was included in both models to account for repeat sampling of individuals (at 0 and 3 h).

4.4 Results

4.4.1 CORT secretion and body condition

Baseline CORT did not significantly vary among populations during either the non-breeding (Fig 4.1a, Table 4.2) or breeding (Fig 4.1a, Table 4.2) seasons. Baseline CORT secretion significantly increased from the non-breeding season to the breeding season in the Stephens (LM, t=3.16, P=0.001, Fig 4.1a) and LA (LM, t= 2.92, P=0.004, Fig 4.1a) populations, but was similar between seasons in the NBI (LM, t= 1.09, P=0.277, Fig 4.1a) and Taranga (LM, t= -0.16, P=0.874, Fig 4.1a) populations.

The CORT response varied significantly among populations during both seasons (Fig 4.1b, Table 4.2). During the non-breeding season, the CORT response was similar between the Stephens and NBI populations (Fig 4.1b, Table 4.2), the Taranga population was significantly higher than the Stephens (Fig 4.1b, Table 4.2) and NBI (LM, t= 3.55, P=0.001, Fig 4.1b) populations, and the LA population was significantly higher than the Stephens (Fig 4.1b, Table 4.2), NBI (LM, t= 5.84, P<0.001, Fig 4.1b), and Taranga (LM, t= 2.79, P=0.006, Fig 4.1b) populations . During the breeding season, the CORT response was similar among the Stephens, LA, and Taranga populations, and was significantly lower in the NBI population (Fig 4.1b, Table 4.2). The CORT response significantly decreased from the non-breeding season to the breeding season in the LA population only (LM, t=-4.38, P<0.001, Fig 4.1b), but was similar between seasons in the Stephens (Fig 4.1b, Table 4.2), NBI (LM, t=-1.27, P=0.205, Fig 4.1b), and Taranga (LM, t= -1.07, P=0.287, Fig 4.1b) populations.

Body condition varied significantly among populations during both seasons (Fig 4.1c, Table 4.2). During the non-breeding season, body condition was significantly higher in the Stephens population compared to all other populations (Fig 4.1c, Table 4.2), was similar between the NBI and Taranga (LM, t= 1.14, P =0.256, Fig 4.1c) populations, and the LA and Taranga (LM, t= -1.10, P = 0.270, Fig 4.1c) populations, and was significantly lower in the NBI population compared to the LA population (LM, t= -2.23, P = 0.027, Fig 4.1c).

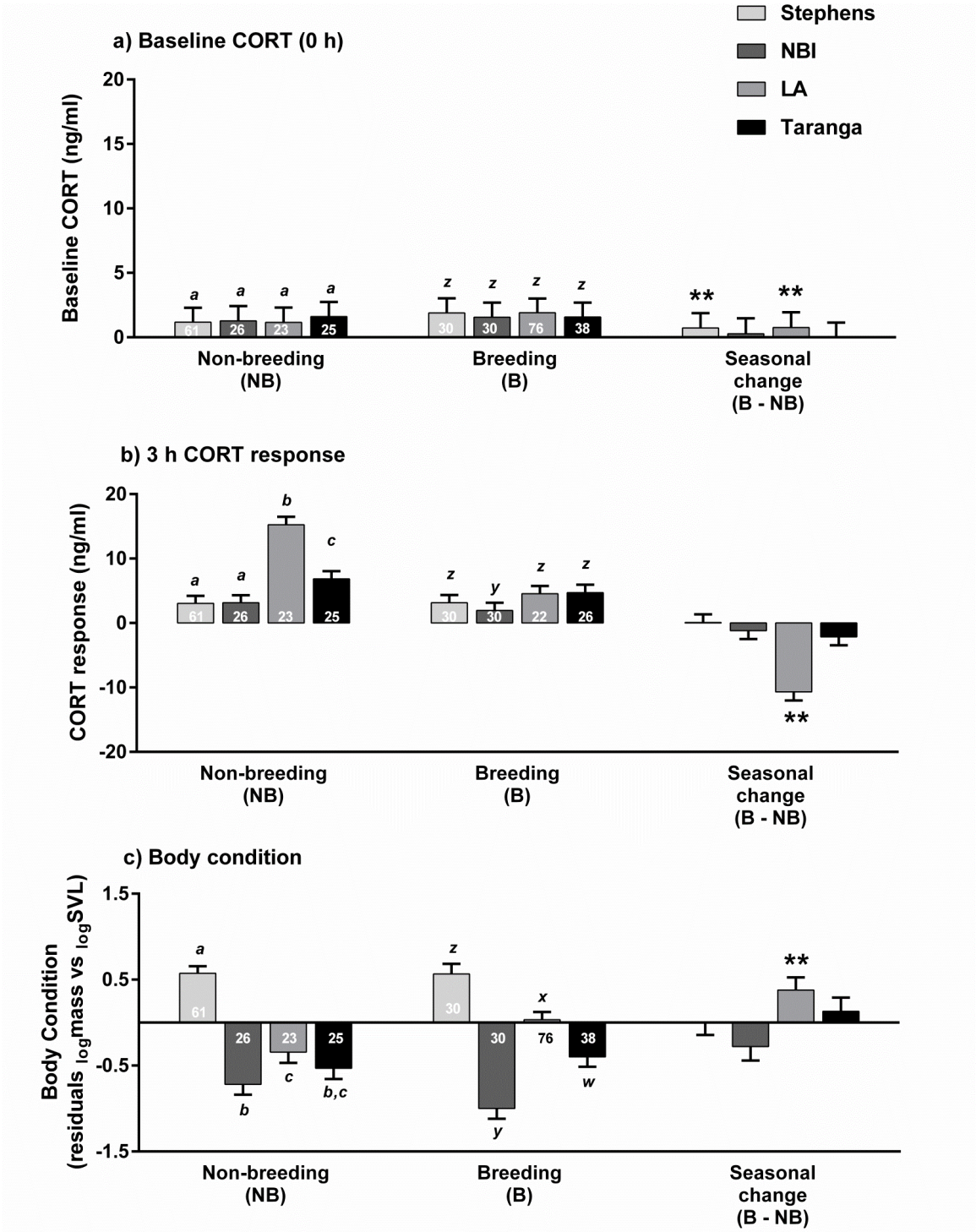

Figure 4.1: Patterns of a) baseline CORT, b) the CORT response (3 h – 0 h CORT), and c) body condition (standardised residuals from regression of TCMlog/SVLlog) among four contrasting populations of adult tuatara (*Sphenodon punctatus*) during the non-breeding and breeding seasons. Data (Mean ± SE) from males and females (non-gravid only) from each population are pooled. Populations sharing the same letters are not significantly different during the non-breeding (*a, b, c*) and breeding (*z, y, x, w*) seasons. Significant seasonal change (breeding season – non-breeding season) is indicated by asterisks ($P<0.01$). Sample size (*n*) is indicated by numbers at base of each bar.

Table 4.2: Linear model parameter estimates for baseline CORT, the CORT response, and body condition examining variation among four populations of tuatara (*Sphenodon punctatus*): Stephens Island (ST), North Brother Island (NBI), Lady Alice Island (LA), and Taranga Island. [1]Centred covariates of body temperature and body condition were included in CORT secretion models.

	Baseline CORT[1]			**CORT response[1]**			**Body Condition**		
Parameter	*Est ± se*	*t*	*P*	*Est ± se*	*t*	*P*	*Est ± se*	*t*	*P*
non-breeding									
(ST - Intercept)	0.06 ± 0.05	1.320	0.187	0.54 ± 0.07	7.781	<0.001	0.57 ± 0.08	6.891	<0.001
season	0.20 ± 0.07	3.162	<0.001***	0.01 ± 0.10	0.079	0.936	-0.01 ± 0.13	-0.053	0.957
NBI	0.03 ± 0.07	0.561	0.575	0.01 ± 0.10	-0.682	0.496	-1.29 ± 0.13	-9.390	<0.001***
LA	-0.01 ± 0.08	-0.070	0.944	0.69 ± 0.12	4.857	<0.001***	-0.91 ± 0.14	-6.363	<0.001***
Taranga	0.13 ± 0.07	1.814	0.070	0.35 ± 0.09	2.957	0.003*	-1.10 ± 0.13	-7.920	<0.001***
breeding									
(ST - Intercept)	0.27 ± 0.06	4.806	<0.001	0.54 ± 0.07	7.700	<0.001	0.57 ± 0.11	4.874	<0.001
season	-0.20 ± 0.07	-3.162	0.001***	-0.01 ± 0.10	-0.079	0.936	0.01 ± 0.13	0.053	0.957
NBI	-0.08 ± 0.08	-1.032	0.303	-0.21 ± 0.12	-2.126	0.034*	-1.56 ± 0.16	-9.749	<0.001***
LA	0.01 ± 0.08	0.080	0.936	0.16 ± 0.11	1.120	0.263	-0.91 ± 0.14	-3.771	<0.001***
Taranga	-0.08 ± 0.08	-0.959	0.338	0.17 ± 0.12	1.210	0.227	-0.96 ± 0.14	-6.129	<0.001***

During the breeding season, body condition varied significantly among all four populations (Fig 4.1c, Table 4.2) with highest to lowest body condition observed in the Stephens, LA, Taranga and NBI populations, respectively. Body condition significantly increased from the non-breeding season to the breeding season in the LA population only (LM, t=2.55, P=0.011, Fig 4.1c), but was similar between seasons in the Stephens (Fig 4.1c, Table 4.2), NBI (LM, t= -1.73, P=0.08, Fig 4.1c), and Taranga (LM, t=0.83, P=409, Fig 4.1c) populations.

4.4.2 Linear ecological predictors of CORT secretion (PCA axes)

I used principal components analysis (PCA) to simplify five ecological variables into linear ecological predictors (Table 4.3). Together, the first two principal components (PC1, PC2) explained approximately 96% of the variance in ecological attributes among the four island populations (Table 4.3).

Table 4.3: Results from principal components analysis (PCA) of five ecological variables from four island populations of tuatara (*Sphenodon punctatus*). Coefficient loadings in bold (≥ absolute value of 0.4) are interpreted as significant.

Ecological Attribute	***Coefficient loadings***	
	PC1	***PC2***
Latitude (deg, min)	**0.450**	-0.142
Tuatara density (#/ha)	**0.400**	**0.440**
Seabird abundance (#/ha)	**0.476**	-0.120
Genetic diversity (H)	0.319	**0.583**
Sex ratio (% males)	0.284	**-0.645**
Importance of components		
Eigenvalues	2.06	1.24
Proportion of variance	70%	26%
Cumulative proportion	70%	96%

In general, the PC1 axis (explaining 70% variance) distinguished an ecological predictor based on habitat variation, in which individuals that loaded positively on

the PC1 axis were from populations at higher latitudes combined with higher tuatara densities and higher seabird abundance (Table 4.3). The PC2 axis (explaining 26% variance) teased out an ecological predictor based on demogenetic variation, in which individuals that loaded positively on the PC2 axis were from populations with a lower percentage of males combined with higher genetic diversity (Table 4.3).

Table 4.4 presents results from the principle component regression analyses of ecological predictors. A significant relationship between baseline CORT and PC1 or PC2 scores was not observed in either season (LM, $P>0.05$, Figs 4.2a and 4.3a). However, a significant negative relationship was observed between the CORT response and PC1 scores during both the non-breeding season (Fig 4.2b, Table 4.4) and (to a greater extent) the breeding season (Fig 4.2b, Table 4.4), and a significant positive relationship was observed between the CORT response and PC2 scores during the breeding season (Fig 4.3b, Table 4.4) but not during the non-breeding season (Fig 4.3b, Table 4.4). In other words, higher CORT responses are associated with a) lower latitude, lower tuatara density and lower seabird abundance (both seasons), and b) balanced sex ratios and greater genetic diversity (breeding season only).

Table 4.4: Linear model parameter estimates for the CORT response in tuatara (*Sphenodon punctatus*) with a fixed effect of season (non-breeding, breeding), and linear covariates of PC1 scores (latitude, tuatara density, seabird abundance) and PC2 scores (% males, genetic diversity) as predictor variables.

Parameter	***Est ± se***	***t***	***P***
(Intercept)	0.78 ± 0.03	27.041	<0.001***
season	-0.04 ± 0.04	-0.899	0.367
PC1	-0.13 ± 0.01	-9.300	<0.001***
season x PC1	0.07 ± 0.02	3.101	0.002**
(Intercept)	0.73 ± 0.03	22.194	<0.001***
season	0.03 ± 0.05	0.639	0.523
PC2	-0.01 ± 0.03	-0.542	0.588
season x PC2	0.13 ± 0.04	3.581	<0.001***

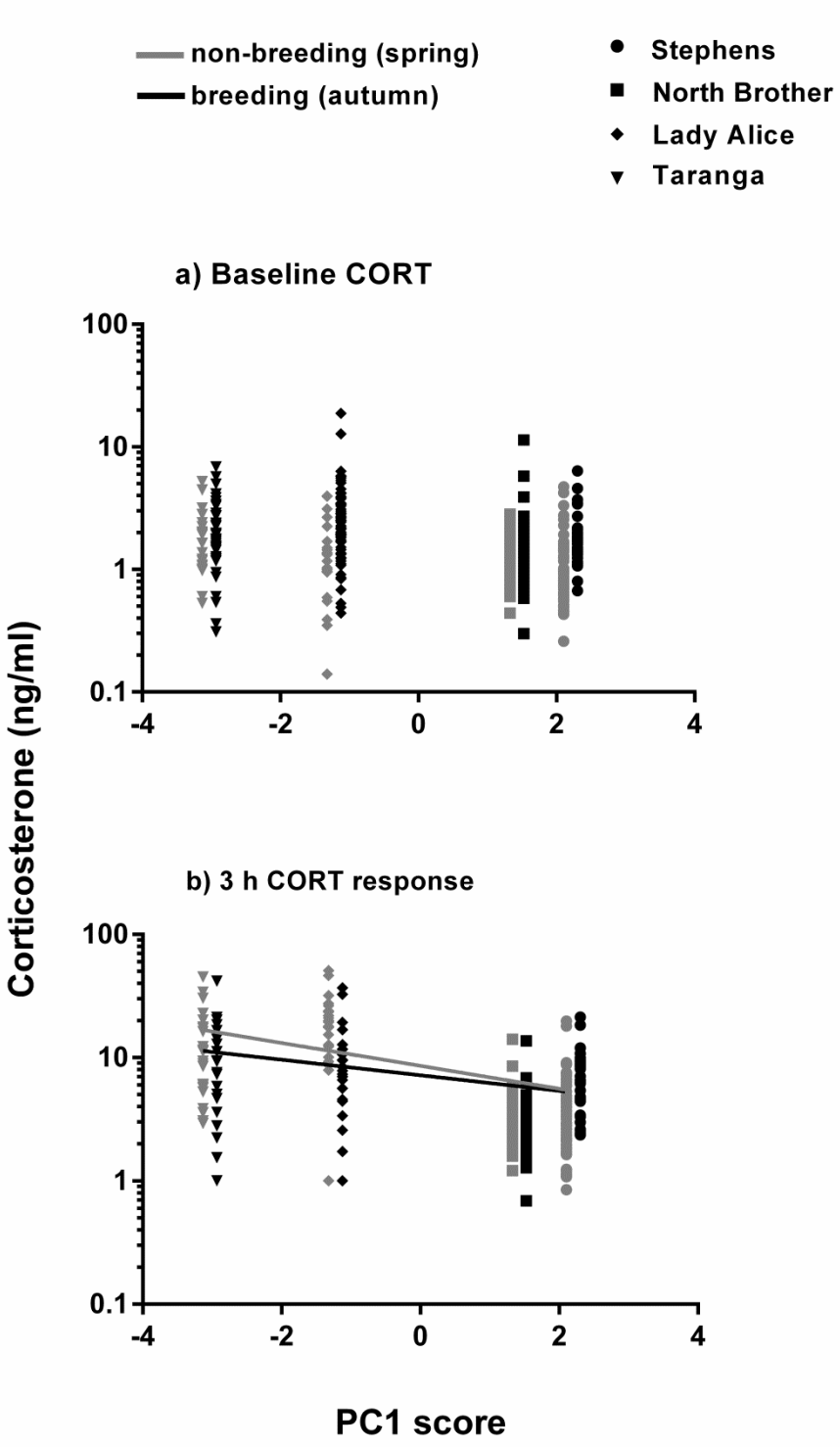

Figure 4.2: The relationship between PC1 "latitude, tuatara density and seabird abundance" on a) baseline CORT and b) the CORT response in four populations of tuatara (*Sphenodon punctatus*) during the non-breeding (gray symbols) and breeding (black symbols) seasons. Individuals with higher PC1 scores are from populations at higher latitudes with higher tuatara density and seabird abundance. A significant relationship was not observed between baseline CORT and PC1 scores. However, a significant negative relationship was observed between the CORT response and PC1 scores during the breeding season (indicated by the solid black line) and to a greater extent during the non-breeding season (indicated by the solid gray line).

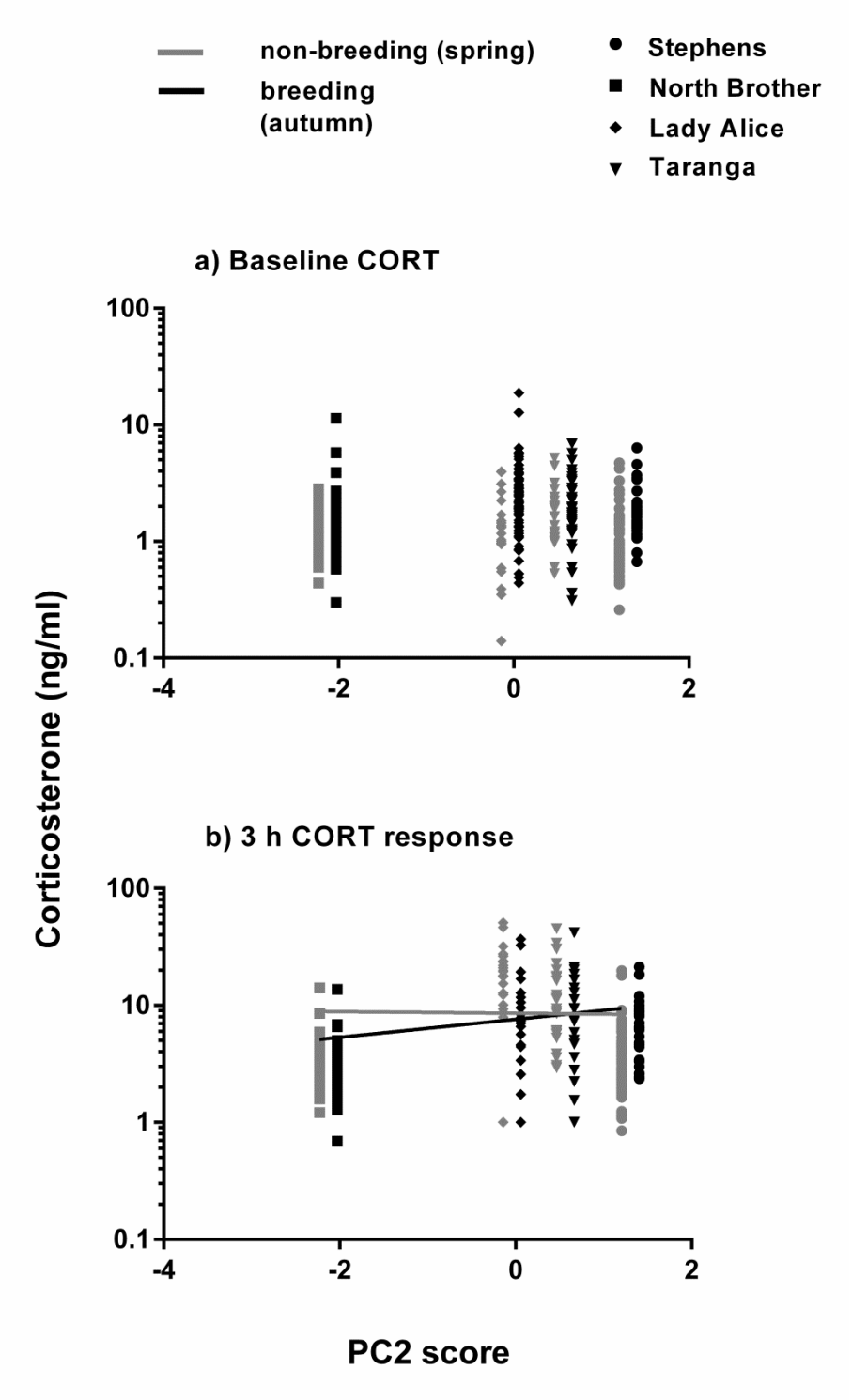

Figure 4.3: The relationship between PC2 "percentage of males in population and genetic diversity" on a) baseline CORT and b) the CORT response in four populations of tuatara (*Sphenodon punctatus*) during the non-breeding (gray symbols) and breeding (black symbols) seasons. Individuals with higher PC2 scores are from populations with higher tuatara density, a lower percentage of males and higher genetic diversity (*H*). A significant relationship was not observed between baseline CORT and PC2 scores. However, a significant positive relationship was observed between the CORT response and PC2 scores during breeding season (indicated by the solid black line) but not during the non-breeding season (indicated by the solid gray line)

4.4.3 Reproductive activity: Testosterone (T) secretion during the breeding season

Testosterone (T) secretion in males varied between the two populations sampled during the breeding season, with males from the Stephens population showing significantly higher T secretion (almost two-fold) compared to males from the NBI population (LMER, t=2.53, P =0.013, Fig 4.4). Testosterone secretion in females was similar between populations (LMER, t=0.65, P=0.517, Fig 4.4). A significant difference in T was observed between sexes in the Stephens population only, with males showing higher T secretion than females (LMER, t=3.83, P<0.001, Fig 4.4). Surprisingly, males and females in the NBI population had similar T secretion (LMER, t=1.87, P=0.065, Fig 4.4). I did not observe a significant T response to 3 h capture-restraint in the Stephens (LMER, t=0.69, P=0.496) or NBI population (LMER, t=-0.37, P=0.713), nor did I find a significant linear relationship between CORT secretion (baseline and/or CORT response) and T secretion (LMER, t =0.33, P=0.743) during the breeding season.

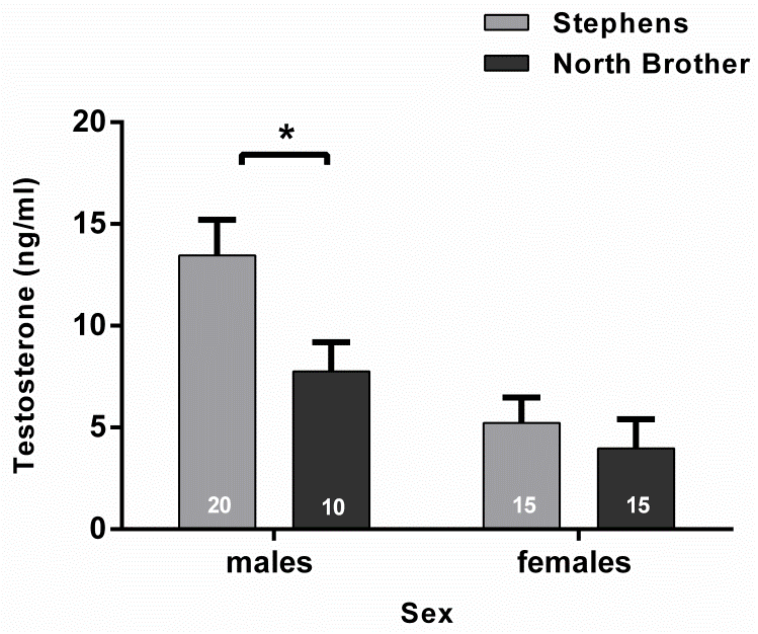

Figure 4.4: Baseline testosterone secretion (mean ± SE) in male and female tuatara (*Sphenodon punctatus*) from Stephens and North Brother populations during the breeding season (autumn). A significant difference between populations is indicated by an asterisk (males only, P=0.013). Sample size (n) is indicated by numbers at base of each bar.

4.5 Discussion

I examined variation in CORT secretion among four populations of a rare reptile (the tuatara, *Sphenodon punctatus*) during the non-breeding and breeding seasons and tested linear ecological predictors of CORT secretion (results summarised in Table 4.5). I established that baseline CORT is similar, whereas the CORT response varies, among populations during each season. Furthermore, I show significant relationships between linear ecological predictors (PCA axes) ant the CORT response but not baseline CORT, suggesting that habitat factors (PC1) and demogenetic factors (PC2) can influence the CORT response. These findings show that the CORT response appears more sensitive than baseline CORT to ecological factors in tuatara, and thus may be a more informative gauge to assess and/or monitor populations.

Secondly, I examined variation in testosterone (T) concentrations between a subset of two populations during the breeding season and examined the association between reproductive activity and seasonal modulation of baseline CORT. I did not find a significant linear relationship between T and CORT secretion during the breeding season; however, significant inter-population variation in T secretion was observed in males, but not females, and may indicate a positive association between male reproductive activity (as indicated by T concentrations) and baseline CORT increase from the non-breeding season to the breeding season.

Table 4.5: Summary of results. (a) Inter-population comparisons for the non-breeding season, breeding season, and seasonal change. Relative levels of inter-population variation of baseline CORT, the CORT response, body condition, and reproductive activity (T) among/between populations are shown. (b) Relationship/association between ecological factors and relevant response variables with direction of trend and associated season(s) shown.

	Inter-population variation			
a) Inter-population comparison	*Baseline CORT*	*CORT Response*	*Body Condition*	*Reproductive Activity (T)*[1]
Non-breeding season (NB)	low	high	high	-
Breeding season (B)	low	low	high	high (males) low(females)
Seasonal modulation (NB to B)	high	low	low	-

b) Relationship/ association	***Response variable***	***Direction***	***Season***
Body Condition	Baseline CORT CORT response	No relationship	Both (NB & B)
Seasonal modulation	Baseline CORT	Positive	NB to B
Habitat effects (PC1) ↑ Latitude ↑ Tuatara density ↑ Seabird abundance	CORT response	Negative	Both (NB & B)
Demogenetic effects (PC2) ↓ Male-bias sex ratio ↑ Genetic diversity	CORT response	Positive	B
Male reproductive activity[1] ↑ T concentrations	Seasonal change (B-NB) in baseline CORT	Positive	B

[1] Stephens and North Brother populations only

4.5.1 The CORT response and body condition vary among populations, but baseline CORT does not

Body condition varied significantly among populations; however, body condition was not a significant predictor of CORT secretion (baseline CORT or the CORT response) in spite of the apparent variation. The lack of relationship may be explained by the fact that a certain threshold must be met for body condition to affect CORT secretion (Romero and Wikelski, 2001).

Contrary to my prediction, baseline CORT did not vary among populations during the non-breeding or breeding seasons. Baseline CORT is often used as an indicator of overall health or stress levels (Bonier *et al.*, 2009) and the CORT response is often used as an indicator of an individual's ability to respond to a stressor (Breuner *et al.*, 2008). The CORT response varied significantly among populations during both seasons. During the non-breeding season, the CORT response was higher in the Lady Alice and Taranga (Northern) populations, compared to the Stephens and North Brother (Cook Strait) populations, which is consistent with my prediction. However, the CORT response was similar between all populations during the breeding season, except for the North Brother population which was significantly lower. My findings show that the CORT response appears more sensitive than baseline CORT to ecological factors in tuatara, and thus may be a more informative gauge to assess and/or monitor populations.

4.5.2 Relationships between The CORT response and linear ecological attributes (PC1 and PC2 scores)

Significant relationships were observed between the CORT response, but not baseline CORT, and linear ecological attributes (PC1 and PC2). Thus, the variation in the CORT response that I observed among populations during the non-breeding and breeding seasons is currently best explained by 1) habitat factors of latitude, tuatara density and seabird abundance (PC1) and 2) demogenetic factors of sex ratio and genetic diversity (PC2).

4.5.2.1 Habitat factors of latitude, tuatara density and seabird abundance (PC1)

A significant positive relationship was observed between PC1 scores and the CORT response during the non-breeding and breeding seasons. The first PCA axis (PC1) had significant positive loadings of latitude, tuatara density and seabird abundance (Table 4.3). In other words, higher PC1 scores are connected to individuals from populations at higher latitudes with higher tuatara density/seabird abundance.

It is possible that tuatara populations at lower latitudes mount a greater CORT response due to the milder climate (where mounting a greater CORT response may be less energetically costly). Warmer but drier climates (not necessarily more benign for tuatara) are experienced at lower latitudes, compared to higher latitudes. Moore *et al.* (2001) found that male garter snakes (*Thamnophis sirtalis*) living at a lower latitude had higher CORT responses than those at higher latitudes, and the authors suggest that snakes inhabiting milder climates (with extended breeding seasons) can energetically afford to mount a greater CORT response. Similarly, a recent study on tadpoles (*Rana temporaria*) found that the CORT response was higher in low-latitude populations, and the authors associated a lower CORT response in high-latitude populations with avoidance of CORT-mediated reduction in growth and development (Dahl *et al.*, 2012). In birds, there is support that populations from higher latitudes exhibit a lower CORT response compared to those in lower latitudes (Hau *et al.*, 2010; Silverin *et al.*, 1997). On the other hand, there are also studies in both reptiles and birds that have found no correlation between latitude and the CORT response (Hews and Baniki, 2013; Quirici *et al.*, 2014).

Lower tuatara density/seabird abundance is indicative of a history of introduced rats (the Pacific rat, *Rattus exulans*) (Cree *et al.*, 1995; Jones *et al.*, 2008; Towns, 2009) and it is possible that past/recent presence of rats has directed selection of a greater CORT response to deal with indirect and direct effects of predation and/or resource competition. Tyrrell *et al.* (2000) observed that tuatara from a rat-inhabited island had significantly higher CORT responses, but similar baseline CORT, compared to tuatara from a rat-free island (Blair *et al.*, 2000). Similarly, higher CORT responses have been observed in marine iguanas (*Amblyrhynchus cristatus*)

exposed to invasive predatory mammals (*Felis catus*, *Canis lupus*) (Berger *et al.*, 2007; Rödl *et al.*, 2007). Therefore, the past/recent presence of an introduced species/predator may induce a long-term effect of sensitisation in stress responsiveness and/or a higher CORT response may be a modulating mechanism to cope with impact of a novel stressor (Berger *et al.*, 2007; Langkilde and Trompeter, 2011; Rödl *et al.*, 2007).

The significant relationship between PC1 and the CORT response was stronger during the non-breeding season (Fig 4.2b), which could indicate that competition for food resources may be greater during the non-breeding season, especially on islands with a history of rats. There is ample evidence that seabirds elevate invertebrate density, and both invertebrates and seabirds (which can be reduced by introduced rats) are an important food source for tuatara in island populations (Markwell and Daugherty, 2002; Towns, 2009; Towns and Broome, 2003; Towns *et al.*, 2007). While I did not collect any quantitative measures of food availability, the seasonal change in body condition observed in the Lady Alice population in this study (in which body condition significantly increased from the non-breeding to the breeding season) provides support for reduced food availability during the non-breeding season.

4.5.2.2 Demogenetic factors of sex ratio and genetic diversity (PC2)

A significant negative relationship was observed between PC2 scores and the CORT response during the breeding season, but not during the non-breeding season. . The second PCA axis (PC2) had a significant negative loading of percentage of males in the population and a significant positive loading of genetic diversity (Table 4.3). In other words, higher PC2 scores are connected to individuals from populations with a lower percentage of males present and higher genetic diversity.

A highly skewed male-biased sex ratio in the North Brother population (Grayson *et al.*, 2014) could indicate more male-male aggressive interactions during the breeding season; therefore, the CORT response may be modulated in tuatara to avoid chronic elevation of CORT. Male tree lizards (*Urosaurus ornatus*) experiencing

a single male-male aggressive encounter experienced a sustained CORT response up to 24 h, but males that continued interacting with other males had a dampened CORT response, which may facilitate metabolic recovery (Knapp and Moore, 1995). In male fruit bats (*Artibeus jamaicensis*), a dampened CORT response during the breeding season could reflect lowered stress sensitivity to avoid chronically elevated CORT levels in times of aggressive and costly male-male encounters (Klose *et al.*, 2006).

There are very few studies examining relationships between genetic diversity and the CORT response, and I found none for reptiles or birds, however; my results are consistent with studies on mammals. Sea otter populations with lower genetic diversity had lower CORT response to capture (Larson *et al.*, 2009). Sarrieau *et al.* (1998) found that genetic diversity had no effect on baseline CORT in rats; whereas, the CORT response was lower in inbred rats compared to outbred and hybrid rats. Clearly, further research examining relationships between demogenetic effects and CORT secretion in reptiles is required to determine whether consistent patterns are seen across species.

4.5.3 Associations between reproductive activity (T) and modulation of CORT secretion

A significant seasonal increase in baseline CORT (from the non-breeding season to the breeding season) was observed in only two of the four populations, which reveals that (contrary to my prediction) seasonal modulation of baseline CORT varies among populations. Seasonal change in baseline CORT was slight in the North Brother population and negligible in the Taranga population. Conversely, the Stephens and Lady Alice populations had significant seasonal change in baseline CORT, with increased secretion during the breeding season.

Numerous studies have reported increased baseline CORT in vertebrates during the breeding season, which is attributed to meeting energetic demands of reproductive activities (Moore *et al.*, 2001; Moore *et al.*, 2000b; Romero, 2002; Wingfield and Sapolsky, 2003). The CORT-fitness hypothesis suggests that the relationship

between CORT secretion and fitness may depend on reproductive activity of individuals (Bonier *et al.*, 2009) and that a seasonal increase in baseline CORT levels is necessary to meet demands of predictable reproductive activities such as gametogenesis, courtship and mating (Bonier *et al.*, 2009; Landys *et al.*, 2006; Whirledge and Cidlowski, 2013). Therefore, variation in seasonal modulation of baseline CORT among populations may be explained by different levels of reproductive activity. In support of this idea, I determined levels of T secretion (as a proxy of reproductive activity) in male and female tuatara from the Stephens population (significant seasonal change in baseline CORT) and the North Brother population (no seasonal change in baseline CORT) to examine the association between seasonal modulation of baseline CORT and reproductive activity.

Testosterone secretion in males from the Stephens population (~14 ng/ml) was nearly twice the level of T secretion observed in males from the North Brother population (~ 7.5 ng/ml) (Fig 4.4). Previously reported levels of T concentrations in male tuatara are ~ 14-16 ng/ml on Stephens Island (Cree, 2014; Cree *et al.*, 1990b) and ~ 10 ng/ml on NBI (Cree, 2014). As expected, I found higher T secretion in males compared to females, but the difference only reached statistical significance in the Stephens population. It is generally recognized that territoriality and other aspects of male mating behaviour are regulated by androgens such as T (Garamszegi *et al.*, 2005; Jessop *et al.*, 1999a; Schramm *et al.*, 1999; Sinervo and Miles, 2011; Wingfield *et al.*, 1990). Given the highly male-biased sex ratio (3.2 males : 1 female) in the North Brother population (Grayson *et al.*, 2014), it is probable that some males have entirely no access to females, as during the breeding season (in any one year) in the North Brother population, it is likely that only a few dozen females are in breeding condition (i.e. sexually receptive) (Mitchell *et al.*, 2010). As male tuatara exhibit highly territorial behaviour (Moore, 2008), a proportion of males in the North Brother population are probably constrained to a 'male-only' environment, which may explain the lower levels of reproductive activity (specifically courting, mating, territorial defence and mate guarding). Research on reproductive activity and mating behaviour in tuatara is currently limited to information collected on Stephens Island; and to my knowledge there have been no studies that examine the association between baseline CORT and male reproductive activity, therefore

studies on mating behaviour/reproductive activity in contrasting ecological scenarios would be required to support this association.

Testosterone secretion varied between populations for males, but not for females. Previous studies have shown that the annual gravidity rate for female tuatara from the North Brother population was half that of the Stephens population (Table 4.1). My results for T secretion in females suggest that the proportion of ovulating females is similar between the Stephens and North Brother populations. Possible explanations could be that female gravidity rates in the North Brother population have increased, that females ovulate but fail to produce shelled eggs, or that females nest earlier (and are no longer gravid when sampled). Alternatively, a sampling bias could have occurred in my study if ovulating (i.e. sexually receptive) females were more readily captured during the breeding season. Nonetheless, inter-population variation in T was observed in males (but not females) and was positively associated (though not statistically tested) with increased baseline CORT from the non-breeding season to the breeding season, suggesting male reproductive activity may drive the seasonal change in baseline CORT.

4.6 Conclusions

Results from this study demonstrate that baseline CORT is relatively stable among populations, despite considerable environmental and genetic variation among populations. However, the CORT response varies and significant relationships between the CORT response and linear ecological predictor are observed, which suggests that stress reactivity is more sensitive than baseline CORT to ecological conditions (such as climate, food resource availability and/or introduced species) and/or demogenetic effects (such as sex ratio and/or inbreeding depression). Future research could tease out the influence of habitat and demogenetic predictors on the CORT response in tuatara, specifically through translocation programs, by examining whether patterns of the CORT response at the population-level are solely a product of selection and are not plastic to changes in the environment.

CHAPTER 5

Moving house: Long-term dynamics of corticosterone secretion are unaltered in translocated populations of a rare reptile (the tuatara, *Sphenodon punctatus*)[1]

5.1 Abstract

Translocations are an important conservation tool used to restore at-risk species to their historical range. Unavoidable procedures during translocations, such as habitat disturbance, capture, handling, processing, captivity, transport and release to a novel environment, have the potential to be stressful for most species. In this study, I examined acute and chronic stress (through the measurement of the glucocorticoid corticosterone - CORT) in a rare reptile (the tuatara, *Sphenodon punctatus*). Here, I found that 1) the acute CORT response remains elevated during the initial translocation process, but does not increase with cumulative stressors, and 2) the long-term dynamics of CORT secretion are similar in translocated and source populations. Taken together, my results show that translocated tuatara are generally resistant to cumulative acute stressors and show no hormonal sign of chronic stress. Translocation efforts in tuatara afford the potential to reduce extinction risk and restore natural ecosystems.

[1] This chapter is based on the following publication with minor modifications: Anderson L, Cree A, Towns D, Nelson (2015) Moving house: Long-term dynamics of corticosterone secretion are unaltered in translocated populations of a rare reptile (the tuatara, *Sphenodon punctatus*). Conservation Physiology

5.2 Introduction

Translocations are human-assisted movements of living organisms from one area to another and are an important tool for conservation efforts and population restoration of species at risk (Armstrong and Seddon, 2008; Ewen *et al.*, 2012; Seddon *et al.*, 2014). The International Union for Conservation of Nature (IUCN) recognises two types of conservation translocation to restore populations, namely (i) reinforcements, in which individuals are released into an existing population of conspecifics to enhance the sustainability of populations, and (ii) reintroductions, in which individuals are released in a historically occupied area in order to re-establish a population after extirpation. Although these type of movements are ultimately aimed at helping species, the translocation process is inherently stressful, as associated procedures such as habitat disturbance, capture, handling, processing, captivity, transport and release to a novel environment are necessary and unavoidable (Dickens *et al.*, 2010; Germano and Bishop, 2009; Parker *et al.*, 2012). In a recent review, Tarszisz *et al.* (2014) identified physiology as a key disciplinary area that is lacking attention in conservation translocations, and also highlighted how physiological data can improve short- and long-term translocation success.

In vertebrates, the stress-response produces a rapid increase in glucocorticoid hormone secretion (corticosterone (CORT) or cortisol) to help individuals cope with immediate stressors (Wingfield *et al.*, 1998); consequently, non-essential processes (such as reproduction and growth) are suspended until homeostasis returns. Although the stress response serves to promote immediate survival, prolonged or sustained CORT secretion (typically expected during translocations) can manifest as 'chronic stress', and is generally considered detrimental to overall health and fitness (i.e. the CORT-Fitness Hypothesis) (Almasi *et al.*, 2013; Bonier *et al.*, 2009; Parker *et al.*, 2012; Sapolsky *et al.*, 2000; Wingfield and Sapolsky, 2003). In a recent review of the associations between stress and movement of animals, Teixeira *et al.* (2007) concluded that stress is a contributing factor to the success or failure of a translocation project. Stress induced by the initial translocation process and relocation to a novel environment increases the vulnerability of individuals to reproductive failure, disease, starvation, predation, and long-range dispersal,

thereby decreasing the chance that individuals will survive and that a self-sustaining population will result (Dickens *et al.*, 2009; Dickens *et al.*, 2010; Parker *et al.*, 2012; Teixeira *et al.*, 2007).

Measuring and monitoring CORT secretion is the most widely used method for assessing stress in vertebrates (Dickens *et al.*, 2010; Sheriff *et al.*, 2011; Wikelski and Cooke, 2006). Although several factors relevant to translocation efforts influence CORT secretion, studies that assess and monitor stress (by way of CORT secretion) throughout and after the translocation process are limited (Germano and Bishop, 2009; Harrington *et al.*, 2013; Tarszisz *et al.*, 2014). Numerous studies have shown that associated procedures commonly applied in translocation programs (such as capture, handling, transport) stimulate a significant stress-response and influence CORT secretion (Baker *et al.*, 2013; Bliley and Woodley, 2012; Fazio and Ferlazzo, 2003; Fazio *et al.*, 2014; Langkilde and Shine, 2006; Narayan and Hero, 2011). Similarly, altered CORT secretion has been associated with variation of environmental factors such as exposure to humans (French *et al.*, 2010; Taylor *et al.*, 2014) and novel predators (Berger *et al.*, 2007; Rödl *et al.*, 2007), change in food availability (Bryan *et al.*, 2014; Kitaysky *et al.*, 2007; Woodley *et al.*, 2003), latitudinal differences (Dahl *et al.*, 2012; Eikenaar *et al.*, 2012; Quirici *et al.*, 2014; Silverin *et al.*, 1997) and habitat type (Bauer *et al.*, 2013; French *et al.*, 2008; Li *et al.*, 2012; Zhang *et al.*, 2011). In addition to experiencing acute stressors during the initial translocation process, translocated individuals released into new environments are faced with several survival challenges (such as finding food, shelter, and avoiding predators); therefore, physiological stress is inevitable (Teixeira *et al.*, 2007).

Here, I examine the acute CORT response and long-term dynamics of CORT secretion through the translocation process in a rare reptile, the tuatara (*Sphenodon punctatus*). Tuatara are a protected reptile endemic to New Zealand and are the only living representatives of the reptilian order Rhynchocephalia (Jones and Cree, 2012). Although tuatara are now considered non-threatened but "at-risk – relict" (Hitchmough *et al.*, 2013), translocations contribute to conservation and ecological restoration efforts and serve to re-establish tuatara within their pre-human range

(Cree, 2014; Hitchmough *et al.*, 2013). In addition to easing extinction pressure, translocations also offer a chance to examine and address relevant research questions (Cree, 2014; Germano and Bishop, 2009; Miller *et al.*, 2012). In 2012, wild tuatara were translocated to six island and mainland sanctuaries from two source populations (Lady Alice Island and Stephens Island/Takapourewa, New Zealand) (Cree, 2014), which presented an excellent opportunity to examine the CORT response to translocation in multiple populations. Previous studies have examined patterns of CORT secretion in tuatara; in general, baseline CORT in tuatara is fairly low (with plasma concentrations typically 2-5 ng/ml), a significant CORT response to capture-restraint is observed, and female reproductive condition, body temperature, and season (but not time of day) are influential factors (Chapter Two; Chapter Three, Chapter Four; Tyrrell and Cree, 1998). I found that baseline CORT was similar among all populations; however, the CORT response varied with latitude, seabird density, sex-ratio and genetic diversity (Chapter Four). Although translocations of tuatara continue to happen, CORT secretion (as an indicator of stress) during and after the translocation process has not been examined. Comparing CORT secretion simultaneously in translocated and source populations of tuatara would allow detection of altered CORT secretion that is correlated with environmental and/or habitat change and that would be an indication of chronic stress.

This study had two aims: first, I examined the acute CORT response in tuatara at different stages of the initial translocation process, and tested the prediction that the acute CORT response would be amplified with cumulative stressors. Second, I tested whether long-term changes in CORT secretion provide evidence of 'chronic stress' in three translocated populations (compared with the corresponding source populations as controls). I predicted that 1) baseline CORT (post-translocation) would be similar among all populations and 2) the CORT response (post-translocation) would be amplified in translocated populations that experienced a marked environmental and/or habitat change (e.g. a greater latitudinal shift). Moreover, body condition indices (mass relative to snout-vent length) in translocated populations (along with source populations as controls) were examined, as chronic stress can influence energy expenditure (Romero, 2002).

5.3 Materials and methods

5.3.1 Study design

I took advantage of two planned translocations in New Zealand (Fig 5.1) to examine short-term and long-term dynamics of CORT secretion in tuatara throughout the translocation process (Fig 5.2).

Figure 5.1: Populations involved in short- and long-term monitoring of physiological data (corticosterone) throughout a conservation translocation programme in New Zealand. Source populations (white dots) and translocated populations (black dots) are shown.

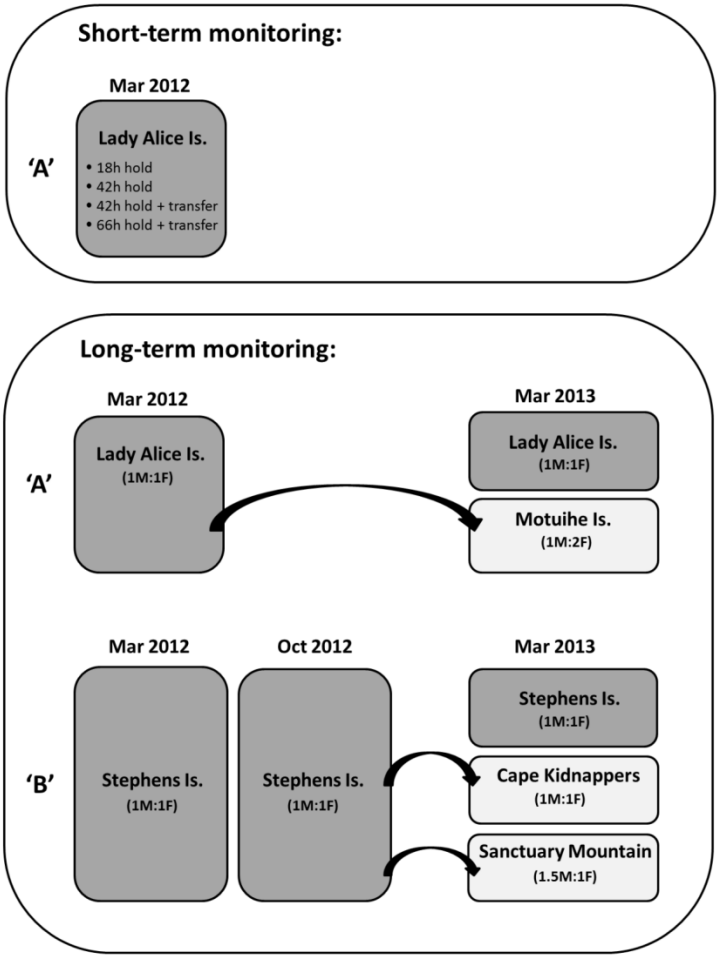

Figure 5.2: Schematic of short-term (upper panel) and long-term (lower panel) monitoring during the translocations to Motuihe Island from Lady Alice Island in March 2012 (Translocation 'A') and to Cape Kidnappers and Sanctuary Mountain Maungatautari from Stephens Island in October 2012 (Translocation 'B'). Short-term monitoring was carried out during Translocation 'A' only. Comparisons for long-term monitoring were made between source (dark gray) and translocated (light gray) populations. Sex-ratio (M:F) for each population is shown.

In translocation 'A' (March 2012) wild adult tuatara were translocated to Motuihe Island (35° 58' S, 174° 43' E) (n=60) from Lady Alice Island (35° 53' S, 174° 43' E). Lady Alice and Motuihe Islands are located in the Northern New Zealand regional climate zone, experiencing sub-tropical warm humid summers and mild winters (NIWA, 2014). In translocation 'B' (October 2012) wild adult tuatara were translocated to five locations from Stephens Island (40° 40' S, 174° 00' E), including Cape Kidnappers Sanctuary (39° 64' S, 177° 09' E) (n=40) and Sanctuary Mountain Maungatautari (38° 30' S, 175° 33' E) (n=50), which were the two locations

monitored in this study. Stephens Island is located in the Northern South Island regional climate zone and Cape Kidnappers Sanctuary is located in the Eastern North Island regional climate zone, both experiencing warm dry summers and mild winters (with frost). Sanctuary Mountain Maungatautari is located in the Central North Island regional climate zone, experiencing warm dry summers and cool winters (with frost and fog) (NIWA, 2014). All translocation release sites offered suitable physical habitat for tuatara (with artificial burrows also provided), and social aspects such as M:F sex-ratio (Fig 5.2) and tuatara densities were within normal range.

5.3.2 Short-term monitoring: The acute corticosterone response at different stages of the initial translocation process

In translocation 'A' only, I examined the acute CORT response (i.e. CORT secretion above baseline) through all stages of the initial translocation process, during which standard translocation protocols were followed (Cromarty and Alderson, 2013; Towns *et al.*, 1990). In summary, adult tuatara (snout-vent length ≥ 170 mm) that were emerged from their underground burrows were captured by hand (between 20:00 h and 04:00 h) and sex was identified by examining secondary sex characteristics such as head size/shape, body shape, spine shape and crest development (Cree, 2014).

All individuals in the translocation programme were subject to a capture-restraint "hold" (which involved capture of individuals and initial holding (between 40 - 60 h) in cloth capture bags), processing (which involved handling, weighing, measuring and implantation of a passive integrated transponder (PIT) tag), and transfer to release site (which involved holding (between 6 -10 h) in perforated cardboard postal tubes (10 cm x 50 cm), movement by foot to the helicopter pick-up site, a 30-min helicopter flight, unloading, and a 30-min handing-over ceremony upon arrival at Motuihe Island). To determine the acute CORT response at different stages of the translocation program, I collected baseline CORT samples (following the blood sampling protocol described in section 5.3.4) from tuatara at capture (0 h) (n=54) and collected a second sample after either (a) an 18 h hold (n=15), (b) a 42 h hold (n=14), (c) a 42 h hold + process + transfer (n=11), or (d) a 66 h hold + process +

transfer (*n*=14). Tuatara do not show significant daily variation in baseline CORT (Tyrrell and Cree, 1998); therefore, time of day at sampling is unlikely to contribute to variation in CORT secretion in this study.

5.3.3 Long-term monitoring: Dynamics of corticosterone secretion post-translocation

Figure 5.2 presents a schematic of samples obtained during translocations 'A' and 'B' and displays sex-ratios (M:F) for each population. For all samples, adult tuatara (both sexes) were captured at night by hand (between 20:00 h and 04:00 h). Upon capture, a baseline CORT sample was obtained, and after 3 h capture-restraint in a cloth bag, a second sample was obtained to determine the CORT response. In translocation 'A', I collected samples from the source population (Lady Alice – LA) prior to translocation (March 2012) and from the source (LA) and translocated (Motuihe – Mot) populations at 12 months post-translocation (March 2013). In translocation 'B', I collected samples from the source population (Stephens – ST) prior to translocation (October 2012) and from the source (ST) and translocated (Cape Kidnappers – CK; Sanctuary Mountain Maungatautari - MT) populations at 6 months post-translocation (March 2013). As significant seasonal variation in CORT secretion has been observed between the breeding (March) and non-breeding (October) seasons in tuatara (Chapter Four), I also analysed samples from the ST source population (obtained in a previous study, March 2012, Chapter Four) for annual comparison. To determine if release-site (within a translocated population) had a significant effect on CORT secretion, I collected post-translocation samples from two separate release-site locations on Mot (Site#1 = Orchards Bush, Site#2 = Von Luckner's Bush) and at MT (Site#1= Tuatarium, Site#2 = Northern Enclosure).

5.3.4 Sampling protocol

To determine baseline CORT concentrations, a blood sample (up to 1 ml) was collected within 10 min of capture from the base of the tail with a heparinized 23-gauge needle and 1 ml syringe. After baseline samples were taken, individuals underwent capture-restraint in a cloth capture bag and/or postal tube (3 h – 66 h depending on study), whereupon a second blood sample (up to 1 ml) was taken to

determine the CORT response. Internal body temperature (T_b) was recorded with a cloacal thermocouple (Fluke® Multimeter, model: 179, specified accuracy ±0.1°C, USA) prior to taking blood samples from each individual (both baseline and CORT response). After CORT response samples were obtained, individual mass (g) was determined (to the nearest ± 5 g) with a 1000 g spring scale (Pesola AG, Switzerland) and snout-vent length (mm), tail length (mm), and tail regeneration length (mm) were measured with a ruler. Body condition scores were generated for each individual as standardized residuals from a regression of log Tail-Corrected Mass (TCM) (Newman *et al.*, 1994) and log SVL (Schulte-Hostedde *et al.*, 2005). Body condition scores were generated separately for each source population (ST and LA) and sex.

Depending on field conditions (i.e. electricity available or not), blood samples were separated either by centrifuge (5 min at 2000 rpm) or under normal gravity for 6 to 8 h at 4°C (Reimers *et al.*, 1983; Sheriff *et al.*, 2011). Plasma was transferred into cryogenic vials with a micropipette, stored in a cryogenic dry shipper (Thermo ScientificTM, Arctic ExpressTM Dual 10) or in a freezer at -20°C until return to the laboratory, and then stored at -80°C until assayed. Corticosterone was analysed with commercial enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI) using a previously described method validated for tuatara (Chapter Two).

Briefly, CORT was extracted from plasma samples with redistilled dichloromethane and each sample was assayed in duplicate. For each extraction, a subset of tritiated CORT samples were analysed to measure extraction recovery. Mean extraction recovery was 106% ± 8% s.d. with an overall CV of 7%. Intra-assay and inter-assay CVs were 9.9% and 14.2%, respectively.

5.3.5 Statistical analyses

Data analyses were carried out using R v3.2.0 statistical software (R Development Core 2013) and Prism 6 (Graphpad Software Inc.). All data were checked for assumptions of normality and were transformed if necessary. Linear mixed effects regression (LMER) models were fitted using the 'lme4' package (Bates, 2013) in R to analyse 1) the acute CORT response during the initial translocation process and 2)

long-term dynamics of CORT secretion in translocated populations. Models were constructed through forward/backward stepwise regression procedures (Field *et al.*, 2012). In all LMER models, a random effect of tuatara ID was included to account for repeat sampling of individuals. The 'lmerTest' package (Kuznetsova, 2013) was used to compute *P*-values for coefficients in final models and significance was assumed at $P < 0.05$.

Sex (M, F) and linear covariates of body temperature (T_b) and body condition score (residuals from $_{log}$Mass vs. $_{log}$SVL) were not significant predictors of CORT secretion in this study (LMER, $P>0.05$), and therefore were not included in final models. Furthermore, the location of release site (within translocated populations) did not have a significant effect on CORT secretion in either translocation study A ($P=0.775$) or B ($P=0.656$), therefore; individuals from separate release sites within translocated populations were pooled for further analyses.

In analysis 1 (short-term monitoring), log transformed CORT was the response variable and sample (baseline, 18 h hold, 42 h hold, 42 h hold + processing + transfer, 66 h hold + processing + transfer) was the input variable. In analysis 2 (long-term monitoring), I first examined whether CORT secretion varied by release site within translocated populations, with log transformed CORT the response variable and input variables of hour (0 h, 3 h), site (#1, #2), and an interaction term of hour x site. Models were fitted to data from Motuihe Island and Sanctuary Mountain Maungatautari, as these translocated populations had two separate release locations. Then, I compared CORT secretion between translocated and source (control) populations, with log transformed CORT the response variable and input variables of hour (0 h, 3 h), sample (source pre-, source post-, translocated pre-, translocated post-) and an interaction term of hour x sample. Lastly, I compared body condition pre- and post-translocation, with body condition score (residuals from $_{log}$Mass vs. $_{log}$SVL) the response variable and sample (source pre-, source post-, translocated pre-, translocated post-) the input variable. Models were fitted to data from translocation 'A' and 'B'.

5.4 Results

5.4.1 Short-term monitoring: The acute corticosterone response during different stages of the translocation process

An acute CORT response (indicated by a significant increase from baseline CORT) was observed in all stages of the translocation process of tuatara from Lady Alice Island to Motuihe Island (Table 5.1, Fig 5.3). The acute CORT response peaked at 18 h hold and successively decreased (though remaining significantly higher than baseline CORT) at 42 h hold (LMER, t=-4.495, P<0.001), 42 h hold + processing + transfer (LMER, t=-3.899, P<0.001) and 66 h hold + processing + transfer (LMER, t=-4.118, P<0.001) (Fig 5.3).

Contrary to my prediction, cumulative procedures of processing + transfer did not amplify the acute CORT response, as individuals held for 42 h (without processing + transfer) showed a similar acute CORT response to individuals held for 42 h + processing + transfer (LMER, t= 0.305, P=0.761) and to individuals held for 66 h + processing + air-transfer (LMER, t= 0.371, P=0.712). Corticosterone concentrations in animals experiencing the latter three treatments were significantly lower than in individuals held for 18 h only (Table 5.1, Fig 5.3).

Table 5.1: Corticosterone secretion (ng/ml) in tuatara (*Sphenodon punctatus*) during different stages of the translocation process. Coefficient estimates (positive or negative) are shown and indicate direction of the linear regression from the intercept (baseline CORT 0 h). Standard errors (s.e.), *t*-values and *P*-values are shown.

Stages of Translocation Process	*estimate*	*s.e.*	*t* value	*P* value
(Intercept)	0.400	0.038	10.39	<0.001
18 h hold	0.956	0.077	12.42	<0.001
42 h hold	0.496	0.079	6.25	<0.001
42 h hold + process + transfer	0.530	0.088	6.01	<0.001
66 h hold + process + transfer	0.535	0.079	6.74	<0.001

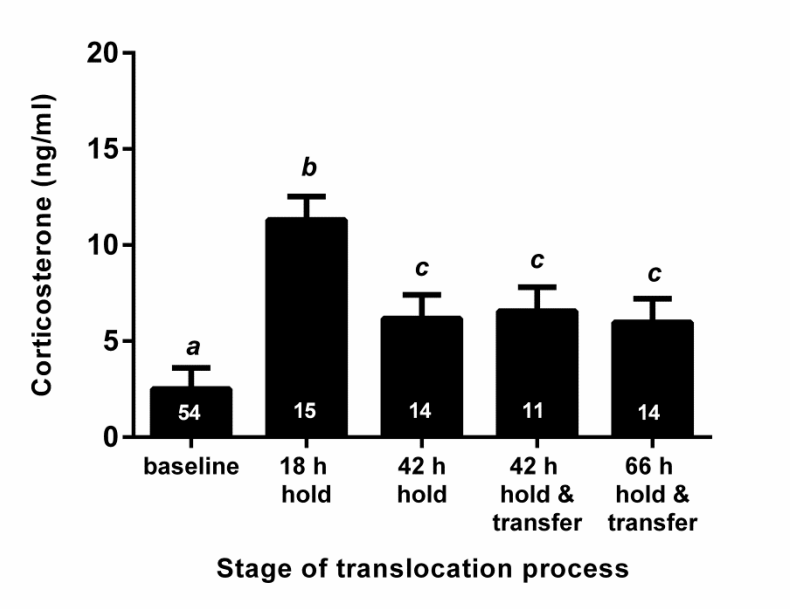

Figure 5.3: Short-term monitoring. The corticosterone response (ng/ml) of tuatara (*Sphenodon punctatus*) at different stages of the translocation process during translocation 'A' to Motuihe Island from Lady Alice Island in March 2012. Sample size (*n*) is indicated by numbers at base of each bar. Bars that share identical letters are not significantly different (*P*>0.05).

5.4.2 Long-term monitoring: Dynamics of corticosterone secretion in translocated populations

In translocation 'A', CORT secretion was similar between translocated (Mot) and source (LA) populations at 12 months post-translocation (Baseline CORT: LMER, t= -0.163, P=0.871; CORT response: LMER, t= 1.136, P=0.259, Fig 5.4a). In both populations, baseline CORT was significantly higher at 12 months post-translocation (March 2013), compared to pre-translocation (March 2012); however, the CORT response was similar pre- and post- translocation (Table 5.2a, Fig 5.4a).

In translocation 'B', CORT secretion varied between translocated (CK and MT) and source (ST) populations at six months post-translocation. Baseline CORT was significantly lower in one translocated population (CK) (LMER, t= -2.345, P=0.020), but was similar in the other translocated population (MT) (LMER, t= -0.925, P=0.356), compared to the source (ST) population (Fig 5.4b). The CORT response was similar in one translocated population (CK) (LMER, t= -1.247, P=0.213), but was significantly higher in the other translocated population (MT) (LMER, t= 1.991,

P=0.048), compared to the source (ST) population (Fig 5.4b). CORT secretion (both baseline CORT and the CORT response) was similar between the two translocated populations (CK and MT) (Baseline CORT: LMER, t= 1.210, P=0.227; CORT response: LMER, t= 0.745, P=0.457, Fig 5.4b).

CORT secretion in the source (ST) population was similar between both pre-translocation samples (Mar '12 vs Oct '12) (Table 5.2b, Fig 5.4b). In all populations, baseline CORT was significantly higher at six months post-translocation (Mar '13), compared to both pre-translocation samples (Mar '12 and Oct '12) (Table 5.2b, Fig 5.4b). The CORT response was similar pre- and post-translocation in the two translocated populations (CK and MT), but was significantly lower post-translocation in the source (ST) population (Table 5.2b, Fig 5.4b).

5.4.3 Body condition

In translocation 'A', body condition was similar between translocated (Mot) and source (LA) populations at 12 months post-translocation (LMER, t=1.342, P =0.183, Fig 5.5a). In both populations, body condition was significantly lower at 12 months post-translocation (Mar '13), compared to pre-translocation (Mar '12) (Mot: LMER, t= -4.632, P<0.001; LA: LMER, t= -7.514, P<0.001, Fig 5.5a).

In translocation 'B', body condition at six months post-translocation was similar in one translocated population (CK) (LMER, t=-1.351, P =0.179), but was significantly lower in the other translocated population (MT) (LMER, t= -3.058, P=0.003), compared to the source (ST) population (Fig 5.5b). Furthermore, body condition in the translocated populations (CK and MT) varied as body condition was significantly lower in the (MT) population, compared to the (CK) population (LMER, t=-4.022, P<0.001, Fig 5.5b). Body condition was similar post-translocation (Mar '13), compared to pre-translocation (Mar '12 and Oct '12), in the translocated (CK) and source (ST) populations, but was significantly lower in the translocated (MT) population (CK: LMER, t=1.261, P=0.210; MT: LMER, t= -3.083, P=0.003; ST: LMER, t=-0.077, P=0.938, Fig 5.5

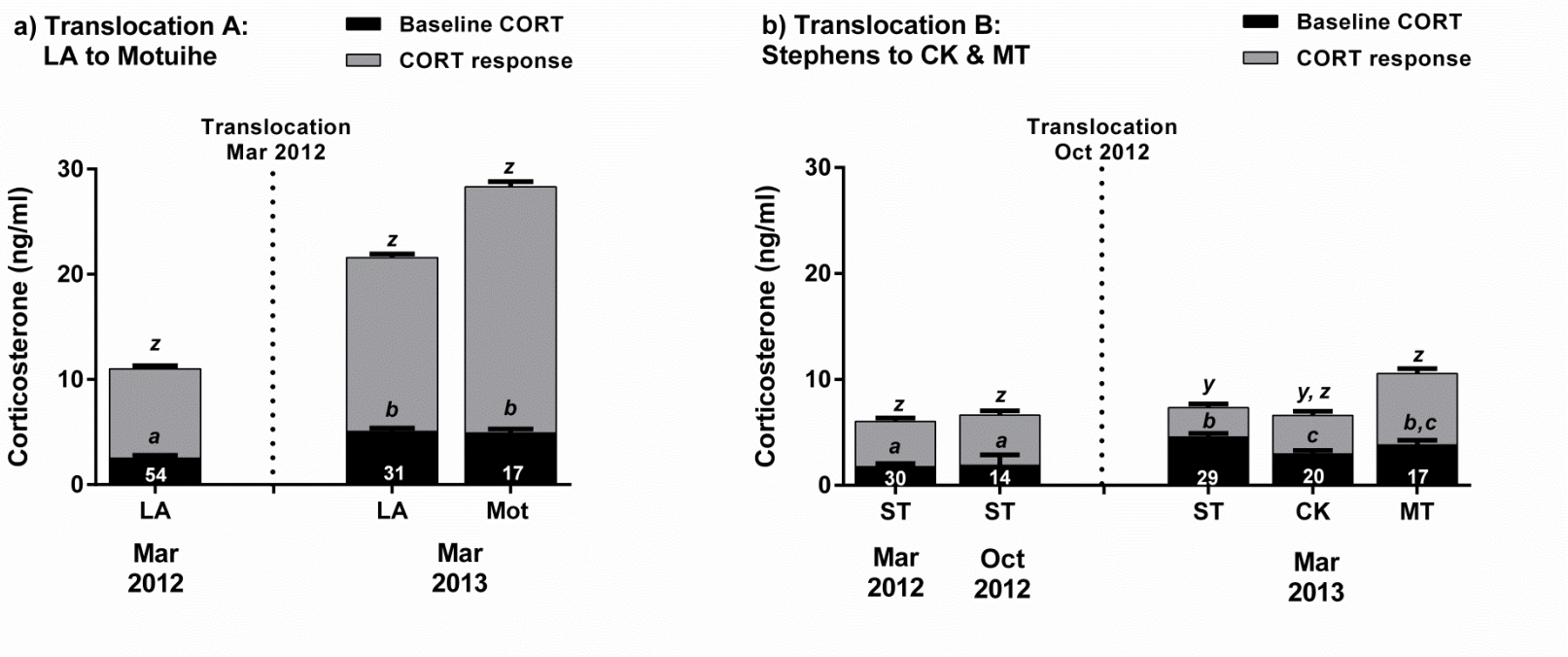

Figure 5.4: Dynamics of corticosterone (CORT) secretion (mean ±standard error) in populations of tuatara (*Sphenodon punctatus*) translocated to a) Motuihe Island (Mot) from Lady Alice Island (LA) and b) Cape Kidnappers Sanctuary (CK) and Sanctuary Mountain Maungatautari (MT) from Stephens Island (ST). Sample size (n) is indicated by numbers at the base of each bar and represents a paired sample of baseline CORT (black bars) and the CORT response (3 h – 0 h) (gray bars) taken from all individuals. Bars that share identical letters are not significantly different (P>0.05).

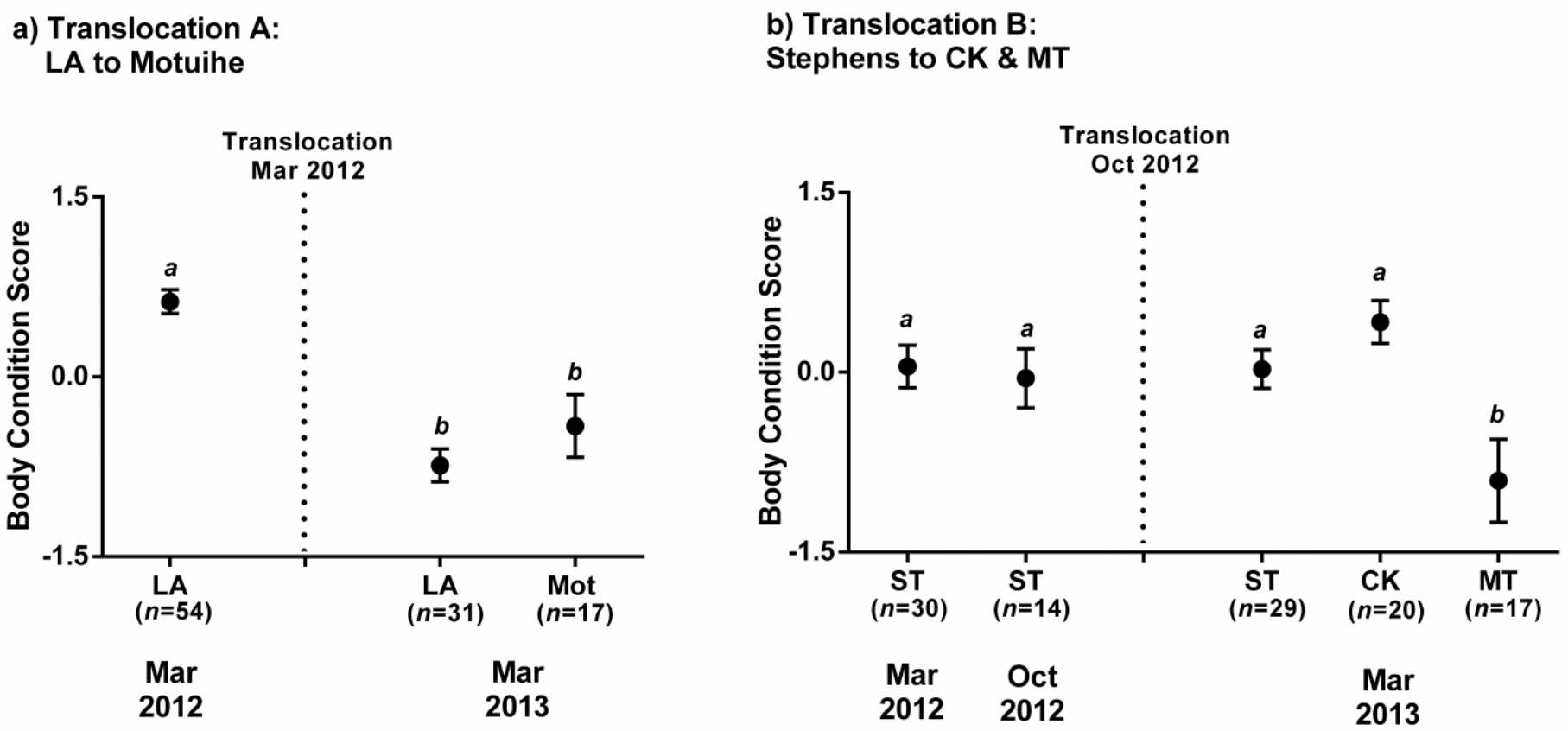

Figure 5.5: Body condition scores (residuals from $_{log}$Mass vs $_{log}$SVL) in populations of tuatara (*Sphenodon punctatus*) translocated to a) Motuihe Island (Mot) from Lady Alice Island (LA) and to b) Cape Kidnappers Sanctuary (CK) and Sanctuary Mountain Maungatautari (MT) from Stephens Island (ST). Data points (mean ±standard error) that share identical letters are not significantly different (P>0.05).

Table 5.2: Results from linear mixed effects regression (LMER) models examining dynamics of CORT secretion (ng/ml) pre- and post- translocation to A) Motuihe Island from Lady Alice Island (source population) and B) Cape Kidnappers and Sanctuary Mountain Maungatautari from Stephens Island (source population). Coefficient estimates (positive or negative) are shown and indicate direction of the linear regression from the intercept (baseline CORT 0 h). Standard error (s.e.), *t*-values and *P*-values are shown.

Long-term CORT dynamics post-translocation	***Est.***	***s.e.***	***t* value**	***P* value**
a) Translocation 'A' Lady Alice (LA) to Motuihe				
(Intercept)	0.401	0.04	9.97	<0.001
hour	0.641	0.05	12.71	<0.001***
post-translocation (LA)	0.306	0.06	4.60	<0.001***
post-translocation (Motuihe)	0.291	0.08	3.55	<0.001***
hour x post-translocation (LA)	-0.013	0.08	-0.15	0.875
hour x post-translocation (Motuihe)	0.115	0.10	1.10	0.271
b) Translocation 'B' Stephens (ST) to Cape Kidnappers (CK) and Sanctuary Mountain (MT)				
(Intercept)	0.244	0.05	4.89	<0.001
hour	0.536	0.07	7.58	<0.001***
pre-translocation (Oct ST)	0.032	0.08	0.36	0.715
post-translocation (Mar ST)	0.414	0.07	5.81	<0.001***
post-translocation (Mar CK)	0.227	0.07	2.88	0.004**
post-translocation (Mar MT)	0.337	0.08	4.05	<0.001***
hour x pre-translocation (Oct ST)	0.008	0.12	0.06	0.945
hour x post-translocation (Mar ST)	-0.329	0.10	-3.26	0.001**
hour x post-translocation (Mar CK)	-0.188	0.11	-1.68	0.093
hour x post-translocation (Mar MT)	-0.093	0.11	-0.79	0.427

5.5 Discussion

Here, for the first time, I examined CORT secretion throughout the entire translocation process in a rare reptile (the tuatara, *Sphenodon punctatus*). I found that 1) plasma CORT concentrations remain elevated throughout the initial translocation process (short-term monitoring between 18 and 66 h), but are not amplified by cumulative stressors and 2) the long-term dynamics of CORT secretion are similar in translocated and source populations. Taken together, my results show that tuatara are generally resilient to cumulative acute stressors and to chronic translocation stress.

5.5.1 Cumulative stressors during translocation do not affect the acute CORT response in tuatara

To my knowledge, this is the first study to quantify the effect of cumulative stressors (routinely experienced in a translocation) on the acute CORT response in a reptile. I expected to see an effect of additive stressors on the acute CORT response, but this was not the case. The CORT response peaked at 18 h of holding/captivity-restraint and additional processing procedures of measurements/microchip insertion and air-transfer did not further increase CORT secretion, suggesting resistance to cumulative stressors in this species. Some species show diel variation of CORT secretion (Breuner *et al.*, 1999; Jones and Bell, 2004) which can confound interpretation of results if samples are not taken at 24 h intervals; however, no evidence of a diel cycle has been found in tuatara (Tyrrell and Cree, 1998). Nevertheless, a significant CORT response was observed throughout all stages of the translocation process, and at no point returned to baseline levels. This observation is consistent with results from my previous study examining the acute CORT response to capture-restraint in tuatara, in which a return to baseline CORT concentrations was not observed over 24 h (Chapter Two). Therefore, I recommend that animal disturbance, holding time, transport duration, and post-translocation disturbance be minimised in tuatara to mitigate potentially harmful effects of sustained CORT secretion in individuals directly following translocation.

In the present study, I did not examine patterns of CORT secretion in the immediate weeks following translocation (my first follow-up sampling occurred at 6 months post-translocation). Consequently, I am lacking information on the speed of recovery to baseline CORT secretion levels. Langkilde and Shine (2006) found that CORT secretion in male and female lizards (*Eulamprus heatwolei*) subject to microchip implantation remained elevated at 14 days (post-treatment), and subsequently increased in response to additional stressors at that time. Similarly, tortoises (*Testudo hermanni*) that experienced handling plus ground transport had increased baseline CORT at four weeks (post-stressor), compared to a control group that only experienced handling (Fazio *et al.*, 2014). These studies have shown that short-term CORT secretion dynamics are significantly altered by processes experienced during a translocation; therefore, obtaining supplementary information on short-term patterns (within four weeks post-translocation) of CORT secretion in tuatara would shed light on the presence of a sustained CORT response and speed of recovery/negative feedback dynamics following translocation.

5.5.2 Long-term dynamics of CORT secretion in tuatara are not altered by translocation

I found that translocation of tuatara did not consistently result in altered CORT secretion relative to controls (source populations) at six or twelve months following translocation (summarised in Table 5.3).

Table 5.3: Summary of long-term dynamics of baseline CORT, the 3 h CORT response, and body condition at 12 months post-translocation in source (Lady Alice Island, Stephens Island – shown in bold) and translocated (Motuihe Island, Cape Kidnappers, Sanctuary Mountain) populations. Arrows indicate direction of change and asterisks denote level of *P*-value significance.

Population	*Baseline CORT*	*CORT Response*	*Body Condition*
Lady Alice Is.	↑ ***	no change	↓ ***
Motuihe Is.	↑ ***	no change	↓ ***
Stephens Is.	↑ ***	↓ **	no change
Cape Kidnappers	↑ **	no change	no change
Sanctuary Mtn.	↑ ***	no change	↓ ***

My results accord with recent studies of translocated reptiles in which CORT secretion was not altered post-translocation. For example, Drake *et al.* (2012) found that baseline CORT in desert tortoises (*Gopherus agassizii*) was similar between translocated and control groups at both one- and two- years post-translocation, and both Holding *et al.* (2014a) and Heiken (2013) found that baseline CORT and the CORT response in translocated northern pacific rattlesnakes (*Crotalus oreganus*) were not altered post-translocation, compared to controls. In contrast, Gerber *et al.* (2004) found that baseline CORT in translocated Turks and Caicos iguanas (*Cyclura carinata*) remained significantly higher than controls at one, five and 12 months following translocation; however, body condition improved and successful reproduction occurred in translocated animals. Although studies are few, my results add to the general reported trend of resilience to translocation and/or translocation stress in reptiles. In contrast, several studies in mammals and birds have reported significant long-term effects of translocation on CORT secretion (Dickens *et al.*, 2009; Franceschini *et al.*, 2008; Gelling *et al.*, 2012; Jachowski *et al.*, 2013; Zidon *et al.*, 2009). However, this observation is not consistent as other studies have reported no long-term effect (Adams *et al.*, 2013; Bosson *et al.*, 2013; Hartup *et al.*, 2005; Ji *et al.*, 2013), suggesting adaptation to new environments (indicated by long-term CORT secretion) is species-specific or context-dependent (e.g. might be due to time of year, weather conditions, or hard- vs soft- release).

Unexpectedly, through long-term monitoring in this study, I observed a significant annual increase in baseline CORT among all source and translocated populations (Fig 5.4, Table 5.3), probably indicating a ubiquitous environmental effect. The CORT response was unaltered in all populations, with the exception of the Stephens Island source population where the CORT response was reduced at 12-months post-translocation (Table 5.3). Body condition declined in the Lady Alice source population and the Motuihe Island and Cape Kidnappers translocated populations. Moreover, these results highlight the importance of collecting information simultaneously from source populations (as a control), as without this my results of increased baseline CORT in all translocated populations, and reduced body condition in two out of three translocated populations, could have been erroneously interpreted as an indication of chronic stress.

It is probable that I detected an unplanned/unexpected effect of drought on baseline CORT secretion in tuatara. In 2012 – 2013, New Zealand experienced its worst drought in 40 years, with the North Island affected more severely (Porteous and Mullan, 2013). Lance *et al.* (2010) observed increased plasma CORT in alligators (*Alligator mississippiensis*) experiencing a severe drought, and recovery of CORT levels (to within normal limits) was observed after substantial rainfall. Although dehydration stress was not directly measured (by way of CORT secretion), Davis and DeNardo (2009) found that water supplementation in a long-lived desert lizard (the Gila monster, *Heloderma suspectum*) led to greater hydration, tail-fat reserves, and surface activity. In a recent experimental study, Dupoue *et al.* (2014) examined CORT secretion in water-deprived snakes (*Antaresia childreni*) and found that the CORT response, but not baseline CORT, was significantly higher in dehydrated snakes, and the loss of body mass was 2-4 times greater, compared to controls. The authors suggest that baseline CORT in snakes may only respond to a more severe degree of dehydration, and that reduced locomotion (to reduce levels of dehydration) may explain the amplified CORT response in water-deprived snakes (Dupoue *et al.*, 2014).

Reptiles, including tuatara, can moderate water loss through behavioural adaptations such as limiting movement/locomotion and retreating to (or not emerging from) burrows, caves, fallen logs, or undersides of rocks where humidity is higher (Bonnet and Brischoux, 2008; Cree, 2014; Davis and DeNardo, 2009; Wilson *et al.*, 2001). Dunlap (1995) found that lizards (*Sceloporus occidentalis*) that were more active (compared to less active) during a drought experienced greater changes in physiological measures (e.g., CORT, weight loss, hematocrit, osmolality). Moreover, Dunlap (1995) suggested that individual variation in behavioural responses of reptiles (e.g. remaining active during drought) can lead to biased analysis of stress in natural populations. Burrowing in tuatara reduces water loss by up to three times the rate experienced when emerged (Cree, 2014). Thus, it is possible that the increased baseline CORT observed in my study is influenced by sampling bias (capturing active individuals out of burrows rather than inactive individuals remaining in burrows).

Contrary to my prediction, a higher CORT response (post-translocation) was not observed in translocated populations experiencing a shift to warmer climates/lower latitudes, specifically the Cape Kidnappers and Sanctuary Mountain translocated populations. In previous studies I observed a higher CORT response in tuatara at higher temperatures (Chapter Three) and at lower latitudes (Chapter Four). The Stephens Island (40° 40') source population showed a reduced CORT response (from pre-translocation to post-translocation), which was not observed in the Cape Kidnappers (39° 64') and Sanctuary Mountain (38° 30') translocated populations (in which the CORT response was unaltered). Similarly, individuals translocated to Motuihe Island (35° 58') from Lady Alice Island (35° 53') did not show an altered CORT response. It is possible that the individuals translocated from Stephens Island (to Cape Kidnappers and Sanctuary Mountain) would have shown a lower CORT response (at 12 months) if they remained on Stephens Island, or were translocated to equal or higher latitudes. Examining CORT secretion in tuatara populations translocated to equal/higher latitudes (e.g. to Orokonui Ecosanctuary (45° 77') from Stephens Island; Cree, 2014), might clarify the effects of latitudinal/climate change on the CORT response.

Although body condition was not significantly correlated with CORT secretion in my study, the sustained body condition in the Cape Kidnappers translocated population, and in the Stephens Island source population, suggests better hydration at these sites in the midst of a drought. Porteous and Mullan (2013) report that New Zealand's South Island (close to where Stephens Island is located) was not affected as severely as the North Island, and better hydration in the Cape Kidnappers population was probably achieved through provision of supplementary water sources (pers. obs.). Reduced body condition has been observed in dehydrated/water-restricted reptiles, including snakes (Dupoue *et al.*, 2014; Lillywhite *et al.*, 2014), lizards (Davis and DeNardo, 2009; Davis and DeNardo, 2010; Dunlap, 1995; Summers and Norman, 1988), alligators (Lance *et al.*, 2010), and turtles (Ray *et al.*, 2004; Ray *et al.*, 2008; van de Merwe *et al.*, 2013). Clearly, information on relationships among water availability/dehydration, body condition, stress, and CORT secretion is lacking, and should be considered in light of imminent climate change.

CHAPTER 6

Corticosterone secretion in tuatara: Thesis summary and conservation applications

6.1 Introduction

The endocrine system in vertebrates produces glucocorticoid hormones (including corticosterone – CORT) that promote basic life processes, regulate life-history transitions and help individuals cope with intrinsic and extrinsic changes/challenges (i.e. stressors); thereby playing an important fitness role (Boonstra, 2013; Busch and Hayward, 2009; Cockrem, 2013). Here, I examined factors that are associated with CORT secretion in tuatara (*Sphenodon punctatus*) and measured CORT secretion as a physiological tool to monitor conservation efforts. Using both observational and experimental studies, I identified intrinsic and extrinsic factors that are significantly correlated with CORT secretion (both baseline and the CORT response) and, for the first time, utilised CORT physiology as a monitoring tool in relevant tuatara conservation efforts (i.e. through a Pacific rat (*Rattus exulans*) eradication and translocation programmes). To date, this thesis delivers the most comprehensive information on CORT physiology in tuatara and provides a foundation for future stress physiology research and baseline data for long-term monitoring of conservation efforts and response to environmental change.

6.2 Summary of findings

The main findings from the previous four data chapters are summarised as follows:

*A) Chapter Two: Modulation of corticosterone secretion in tuatara (***Sphenodon punctatus***): Evidence of a dampened stress response in gravid females*

Sex is not correlated with CORT secretion (either baseline CORT or the CORT response to capture-restraint) in tuatara; however, female reproductive condition is. Gravid females have significantly higher baseline CORT, and a significantly dampened CORT response, compared to non-gravid females (and also males) during the nesting life-history stage. This result supports my hypothesis that gravid females will have a dampened CORT response compared to non-gravid females. The functional significance of elevated baseline CORT secretion in gravid females during nesting is unclear; therefore, experimental studies involving hormone manipulation (in which CORT secretion is increased or decreased) in gravid females would help determine if gravid females are able to mount a significant CORT response (examined in Appendix A; a question re-visited in Chapter Three) and/or if elevated baseline CORT is linked with timing of oviposition (examined in Appendix A).

*B) Chapter Three: Body temperature is correlated with the corticosterone stress response in tuatara (***Sphenodon punctatus***)*

The CORT response is positively correlated with internal body temperature (T_b) in male and female (gravid and non-gravid) tuatara, whereas baseline CORT is positively correlated with T_b in gravid females only. Acute increase in mean T_b 12.0 ± 0.7°C to 21.4° ± 0.4°C elicits a significant CORT response in gravid female tuatara (proving that gravid females are in-fact able to mount a further CORT response – a question posed in Chapter Two) and may increase the CORT response in males and non-gravid females. These results support my hypothesis that an acute increase in T_b increases the CORT response to capture-restraint in tuatara.

*C) Chapter Four: The corticosterone stress response varies among island populations of tuatara (***Sphenodon punctatus***) and is associated with linear ecological attributes*

Baseline CORT was similar among tuatara populations during the non-breeding season and the breeding season (which is contrary to my prediction that baseline CORT varies among populations); however, the CORT response to capture-restraint varied significantly among populations. In general, baseline CORT increased, and the CORT response decreased, from the non-breeding season to the breeding season. This result supports my hypothesis that baseline CORT is higher during the breeding season, compared to the non-breeding season.

Habitat factors of latitude, tuatara density and seabird abundance (PC1 axis) and 2) demogenetic factors of sex ratio and genetic diversity (PC2 axis) are significantly correlated with the CORT response, but not baseline CORT. The correlation with habitat factors provides support for my hypothesis that the CORT response is higher in northern populations that experience a milder climate and the past/recent presence of Pacific rats (*Rattus exulans*).

Testosterone (T) secretion was not directly associated with CORT secretion; however, inter-population variation in T was observed in males (but not females) and was positively associated with increased baseline CORT secretion from the non-breeding season to the breeding season, suggesting male reproductive activity may drive the seasonal increase in baseline CORT.

D) Chapter Five: Moving house: Long-term dynamics of corticosterone secretion are unaltered in translocated populations of a rare reptile (the tuatara, **Sphenodon punctatus***)*

The CORT response to capture-restraint remains elevated during the initial translocation process, but does increase with cumulative stressors. The long-term dynamics of CORT secretion are similar in translocated and source

populations. Translocated tuatara are generally resistant to cumulative acute stressors and show no hormonal sign of chronic stress. These results do not support my hypothesis that cumulative stressors (experienced during the translocation process) and translocation to a novel location increase CORT secretion. It is hypothesised that drought leads to increased baseline CORT secretion in tuatara.

6.3 Conservation implications: The value of CORT as a conservation physiology tool in tuatara

Endocrine techniques have been utilised in ecological studies as a tool to monitor or assess an individual's physiological response to challenges and/or changing environments (Wikelski and Cooke, 2006), and hold great potential to inform and enhance conservation science (Tarszisz *et al.*, 2014). The results of this thesis provide valuable information on intrinsic and extrinsic factors that are associated with CORT secretion in tuatara, which is a necessity when utilising endocrine data as an applied conservation physiology tool.

6.3.1 Patterns of CORT secretion indicate a physiological response to challenges in tuatara

Although there is still much to learn about CORT secretion in tuatara (and reptiles in general), this thesis has indicated patterns in baseline CORT and the CORT response in tuatara in response to challenges/stressors:

6.3.1.1 Baseline CORT

In accordance with other vertebrate species (Hamann *et al.*, 2002; Romero, 2002), gravid female tuatara show elevated baseline CORT secretion during the energetically demanding nesting life-history stage (Chapter Two). Similarly, baseline CORT secretion increases (in both males and females) from the non-breeding season to the breeding season (Chapter Four), which suggests an increase in reproductive

activity (possibly driven by males) (Chapter Four). Baseline CORT likely increased in response to drought conditions and this response was observed among all populations studied here (Chapter Five).

6.3.1.2 The CORT response

Tuatara exhibit a significant CORT response to capture-restraint (Chapter Two – Chapter Five; Cree and Tyrrell (2001)), with restraint times ranging from 1.5 h to 66 h. Throughout all periods of capture-restraint, CORT secretion remained significantly higher than baseline CORT (Chapter Two – Chapter Five).

Cumulative stressors (including handling/measuring, PIT-tagging, extended holding, and helicopter transfer) did not increase the CORT response – which is contrary to what I predicted (Chapter Five), but internal body temperatures (T_b) approaching 21.4°C elicit a significant CORT response in gravid females (Chapter Three). A significant CORT response observed in gravid females (in response to increased T_b/capture-restraint) demonstrates that gravid females are indeed able to mount a CORT response during the nesting life-history stage, which under standard capture-restraint conditions is dampened (Chapter Two).

A significant difference in CORT secretion was not observed in response to the recent Pacific rat (*Rattus exulans*) eradication on Taranga Island (Chapter Four), but my results show that populations that have experienced rats (such as Lady Alice and Taranga Islands) have higher CORT responses compared to populations that have not (such as Stephens and North Brother Island). This result could also be explained as a latitudinal pattern; therefore, further research and/or monitoring of CORT secretion in populations that have experienced Pacific rat introduction/eradication are needed to clarify my results.

The North Brother Island population shows a lower CORT response compared to the other populations studied here, which could be a product of low genetic diversity and/or reproductive activity (Chapter Four). Variation in the magnitude of the CORT response among populations does not necessarily imply that certain environments are more "stressful" than others (Breuner *et al.*, 2008; Breuner *et al.*,

1999), rather the CORT response is considered to be a measure of an individual's ability to respond to a stressor and individuals in certain populations may simply have a modulated CORT response to adaptively cope with their respective environments. It is likely that variation in the numerous regulatory mechanisms of the HPA axis (stress reactivity, negative feedback, regulation of CORT binding proteins, and adrenal sensitivity) are heritable and linked to variation in fitness (MacDougall-Shackleton *et al.*, 2013).

6.3.2 CORT secretion is a valuable addition to the conservation 'tool-box' for tuatara

Results presented in this thesis can be applied toward conservation efforts of tuatara in several ways. The knowledge that female reproductive condition (gravid vs. non-gravid, Chapter Two) and season (breeding vs. non-breeding, Chapter Four) are associated with CORT secretion emphasises the importance of identifying/considering reproductive status/activity. Although testosterone (T) and CORT are not significantly correlated (Chapter Four), seasonal modulation of CORT may inform researchers/managers on the overall reproductive activity level (T) of populations.

Conservation efforts such as population monitoring, research programmes, translocation and captive management programmes routinely employ capture-restraint protocols. These protocols induce a significant CORT response and if carried out for longer time periods (such as in translocations) can constrain normal thermoregulatory behaviours. Knowing that temperature is positively correlated with the CORT response (Chapter Three) informs researchers/managers that caution should be taken when capture-restraint protocols are employed, specifically when longer restraint periods/higher ambient temperatures (>20°C) occur. Potential solutions to offset a higher CORT response include: ensuring cool holding conditions, providing shaded holding areas during translocation/research programmes, dampening catch bags if required.

Knowing that CORT responses are significantly correlated with ecological attributes experienced by specific populations (Chapter Four) informs managers on the

population's adaptive response/ability to respond to stressors, which may come in to play when selecting individuals and/or populations for captive breeding or translocation programmes. For example, in a new environment (considered more benign compared to the original environment), a lower CORT response may be advantageous; however, if the new environment is degraded (i.e. wild to captivity) or becomes unpredictable (i.e. warmer temperatures/susceptible to drought/exposure to predators) a greater CORT response may be advantageous for overall fitness (MacDougall-Shackleton *et al.*, 2013). The merit of a lower versus a higher CORT response is an area of research that requires further testing in tuatara (and vertebrates in general).

The lack of apparent chronic stress in translocated populations of tuatara (Chapter Five) provides positive feedback for ongoing conservation translocation efforts in New Zealand. Moreover, the circumstantial evidence of increased baseline CORT in response to drought (Chapter Five) leads to a hypothesis that requires further testing (discussed further in section 6.4) and highlights the potential value of CORT physiology as a tool to quantitatively monitor the physiological response to impending environmental change (i.e. climate change).

6.4 Recommendations for future research

This thesis provides the most complete picture of CORT physiology in tuatara to date, but several new research topics (and associated questions) have arisen. A solid foundation has been laid for future research examining more complex details of CORT physiology in tuatara, which will expand on the work presented here.

Topic 1: Relationships between CORT secretion and reproduction

- What is the functional significance of elevated baseline CORT and/or a dampened CORT response in gravid females during nesting?

If there is a relation between baseline CORT secretion in gravid female tuatara and timing of oviposition, then manipulation of CORT concentrations (increased or decreased CORT) will induce or delay oviposition. Appendix A presents results from the first hormone manipulation studies investigating modulation of CORT secretion in tuatara (prompted by results obtained in Chapter Two). Unexpectedly, hormone treatments (ACTH to increase CORT secretion; metyrapone to inhibit CORT secretion) did not significantly influence the CORT response to capture-restraint in gravid and non-gravid females compared with saline controls, though the trend of the CORT response for both treatments (in relation to saline controls) was as predicted, and oviposition patterns are suggestive of a functional role for CORT in timing of oviposition (Appendix A). My preliminary results from this experiment can serve as baseline information for future hormone manipulation studies in tuatara, but further studies will be necessary to fine-tune dosage information. It would be useful to carry out a dose-response study for ACTH and metyrapone administration with several response sampling times in order to determine suitable dosages and durations to observe significant effects (Appendix A).

- Does CORT secretion in gravid females vary based on nesting experience (higher/lower for first-time nesters?). Does density of females on nesting grounds/in captivity influence baseline CORT and/or the CORT response?

  It would be useful to determine the influence of nesting experience and density on patterns of CORT secretion in gravid females, as this may explain individual-/population-level variation observed among gravid females (Jessop et al., 1999b; Riechert et al., 2012).

- Do seasonal patterns of CORT reflect levels of reproductive activity?

  If reproductive activity is related to seasonal modulation of baseline CORT in tuatara, then populations with low levels of reproductive activity (as indicated by testosterone (T) in plasma samples) will lack seasonal change

(i.e. increased baseline CORT secretion from the non-breeding season to the breeding season) in baseline CORT. It would be useful to determine levels of testosterone (T) in plasma samples from Taranga to see if patterns are similar to those observed on North Brother Island (Chapter Four). Low concentrations of T on Taranga would suggest low levels of reproductive activity (for both males and females), which may be a product of Pacific rat presence and/or aged individuals on Taranga (Chapter Four).

Examining the above questions would inform future studies investigating whether and/or how CORT plays a role in oviposition/nesting behaviour and reproductive activity in tuatara, and may inform captive management programmes and island restoration programmes where groups of tuatara fail to reproduce.

Topic 2: Exploring relationships between CORT and fitness measures

Future research addressing the following questions will provide understanding and insight towards the effect (if any) of CORT secretion on fitness-related performance measures in tuatara.

- What are the fitness implications (if any) of elevated baseline CORT and/or a dampened CORT response in gravid females? Is there evidence of maternal effects: Is maternal CORT transferred to egg yolk, and if so what are the patterns? Does maternal CORT influence hatchling fitness (i.e. size, growth rate, locomotor performance)?

- What are the fitness implications of increased CORT secretion (as was observed in a drought year – Chapter Five)? What are the relationships between CORT secretion and immune function (i.e. wound healing)? Is there a threshold where body condition influences CORT secretion, as has been seen in marine iguanas (Romero and Wikelski, 2001)?

- What is the relationship between individual genetic diversity and the CORT response? North Brother individuals with less genetic diversity may not respond

as well to stressful environments as those with higher genetic diversity, as has been seen in mammals (Larson *et al.*, 2009; Sarrieau *et al.*, 1998) and invertebrates (Freitak *et al.*, 2014; Reed *et al.*, 2003).

- What factors influence other aspects of the HPA-axis (such as negative feedback efficiency) and what is the relationship (if any) with fitness-related performance measures?

  Variation in negative feedback efficiency (which modulates CORT secretion – Chapter One, Fig 1.1) determines the magnitude and duration of CORT secretion (both baseline CORT and the CORT response to capture-restraint). For example, longer (i.e. older) red-sided garter snakes (*Thamnophis sirtalis parietalis)* (Moore *et al.*, 2000a) and degus (*Octodon degus)* in lower quality habitat (Bauer *et al.*, 2013) both exhibit reduced negative feedback efficiency of CORT secretion.

  It would be useful to test negative feedback efficiency in tuatara, by way of a dexamethasone challenge (Romero and Wikelski, 2006), in individuals of a known age and/or in contrasting environmental situations (both in natural conditions and in a controlled laboratory setting). Results from such experiments would clarify whether age (suggested of Taranga individuals, Chapter Four) or resource competition/habitat quality (suggested of North Brother individuals, Chapter Four) explains inter-population variation in CORT secretion by way of a reduced negative feedback mechanism.

Topic 3: Non-invasive measures of CORT in tuatara

Obtaining blood samples from wild and captive individuals is considered invasive (Sheriff *et al.*, 2011). Several non-invasive measures have been developed to assess/determine CORT in other taxa. Examples include: urinary samples in amphibians (Kindermann *et al.*, 2012; Narayan *et al.*, 2011b), faecal samples in elephants (Jachowski *et al.*, 2013) and horses (Ji *et al.*, 2013), skin sheds in snakes

(Berkvens *et al.*, 2013), claw trimmings in turtles (Baxter-Gilbert *et al.*, 2014), hair samples in bears (Bechshøft *et al.*, 2012), and feather samples in birds (Bortolotti *et al.*, 2008).

Development and validation of non-invasive measures of CORT secretion for tuatara (for which blood collection training/equipment is not required) would allow for collection of opportunistic samples by management/conservation staff in captivity and in the wild. In captive-breeding facilities/captive collections, samples could be collected from individuals to monitor baseline CORT non-invasively, and analysed for relationships with other measures of fitness (size, growth rate, locomotor performance, wound healing). Development of non-invasive CORT determination would also allow for analysis of younger/smaller age classes (as blood sample collection is difficult in hatchlings and juveniles due to their small size), which is an area that has to date gone un-studied with respect to patterns of CORT secretion.

6.5 Summary

The field of conservation physiology is a relatively new discipline, and in order to reliably to use CORT as a conservation tool, one must know before-hand what factors contribute to CORT secretion to effectively control for covariates and interpret results (Dantzer *et al.*, 2014; Wikelski and Cooke, 2006). Measurements of CORT can provide quantitative information about how environmental challenges or stressors impact individuals and populations. Furthermore, monitoring CORT has potential to be used as an 'early warning system' of possible population decline, and threshold levels of CORT could be set and (if reached) prompt implementation of management plans and conservation programmes (Dantzer *et al.*, 2014).

To date, this thesis delivers the most comprehensive information on stress physiology in tuatara, an iconic protected reptile. Intrinsic and extrinsic factors associated with CORT secretion are identified, patterns of CORT secretion in response to challenges/stressors are observed, and the potential value of measuring CORT secretion as a conservation tool in tuatara is explored. These results provide a foundation for future research that will further advance the comparative

understanding of stress physiology in reptiles. More importantly, understanding stress physiology may be critical for managing future population viability of tuatara in a changing climate, where increased temperatures, changes in water availability, and habitat alteration will present challenges.

Appendix A: Hormone manipulation studies in gravid tuatara

Introduction

Gravid females have significantly higher baseline CORT during the nesting life-history stage compared with non-gravid females and males (Chapter Two). Baseline CORT is highest during nest digging and oviposition (Cree and Tyrrell, 2001). Interestingly, a distinct fall (~5-fold) in baseline CORT is observed after oviposition, even in the case of females that continue to guard their nests, which suggests a potential role in the timing of oviposition (Cree and Tyrrell, 2001). Nesting is separated by 6-7 months from mating and ovulation in tuatara (Cree, 1994); therefore, patterns in CORT secretion that are related specifically to nesting/oviposition are much more readily identified than in other reptiles.

In Chapter Two, I tested for (and confirmed), a dampened CORT response in gravid female tuatara during the nesting life-history stage. Here, I further examine the responsiveness of gravid females to adrenocorticotrophic hormone (ACTH) and metyrapone challenges during the nesting life-history stage. The pituitary hormone ACTH stimulates CORT release from the adrenal glands, whereas metyrapone is a CORT-synthesis blocker. Both have been used in several vertebrate species, including reptiles, to manipulate CORT synthesis (Cartledge and Jones, 2007; Dixon *et al.*, 1985; Klukowski, 2011; Thaker *et al.*, 2010; Yang and Wilczynski, 2003). I predicted that CORT secretion would be increased in individual tuatara treated with ACTH and inhibited in individuals treated with metyrapone. A reduced response to ACTH by gravid females compared with non-gravid females would suggest that the ability to secrete CORT is reduced during the nesting life-history stage. In exposing tuatara to these challenges, I also aimed to test whether increased or decreased CORT secretion influences oviposition in gravid females during nesting.

Material and methods

Wild adult tuatara (*Sphenodon punctatus*) were captured and sampled on Stephens Island/Takapourewa (40° 40' S, 174° 00' E) in Cook Strait, New Zealand during the October 2012 nesting season. Emerged individuals were caught by hand at night between 20:00h and 05:00h and were subsequently assigned to an appropriate group based reproductive status (gravid female or non-gravid female). Gravid females were selectively captured at or near a nesting rookery and female reproductive status was inferred through abdominal palpation for shelled eggs.

Studies investigating adrenocortical stress-responsiveness use physiological challenges to confirm that individuals can mount a stress response (Romero, 2002). In this study, gravid female (n=5) and non-gravid females (n=5) were subject to an ACTH challenge to determine if gravid females are capable of mounting a CORT response during nesting. In conjunction, I administered a metyrapone challenge to different gravid (n=5) and non-gravid females (n=5) to test whether increased or decreased CORT secretion influences oviposition. Saline was administered to other gravid (n=5) and non-gravid (n=4) females as a control treatment.

Capture/sampling occurred over 5 nights during the 2012 nesting season (16–20 October). Reproductive condition of females was inferred by abdominal palpation and baseline blood samples were collected within 10 minutes of capture, as described in Chapter Two. Body mass (g) was measured to calculate hormone dosage specific to mass. Individuals were randomly assigned to a treatment of either ACTH (0.03 IU/µl/g body mass; Sigma A6303, fragments 1-39 porcine, Sigma-Aldrich, NZ), metyrapone (30µg/µl/g body mass; 96% 2-methyl-1,2-di-3-pyridyl-1-propanone, Sigma-Aldrich, NZ) or 0.9% saline (1µl/g of body mass). These dosages were chosen on the basis of comparable dosages in other reptile species determined to be effective in producing CORT responses within normal physiological limits (Cartledge and Jones, 2007; Klukowski, 2011; Preest *et al.*, 2005; Romero and Wikelski, 2006; Scholnick *et al.*, 1997; Yang and Wilczynski, 2003). Hormone solutions were freshly prepared at the field site and were held at 4°C until

administered. Treatments were injected intraperitoneally with a 25 G needle and 1 ml syringe. Individuals were subsequently subject to 3 h of capture-restraint stress in individual cloth bags and upon completion were re-bled to determine the effect of capture-restraint, injection and hormone treatment on CORT secretion. CORT concentrations were determined by EIA (as described in Chapter Two).

To test if stimulation (ACTH) or inhibition (metyrapone) of CORT secretion influences oviposition during nesting, I held all individuals in clean cardboard boxes (a standard protocol for induction studies in tuatara (Cree *et al.*, 1991a)) for five days following injection and blood sampling. For the duration of holding, individuals were checked daily for signs of oviposition and daily measurements of body mass (g) and skin temperature (°C) of the dorsal body were taken with a 1000 g spring scale and handheld infrared temperature gun (Hare *et al.*, 2007), respectively, between 14:00h and 16:00h.

Data analyses were carried out using R v3.0.0 statistical software (R Development Core 2008) and Prism 6 (Graphpad Software Inc.). All data were checked for normal distributions and homoscedasticity, and if necessary, were transformed to meet assumptions for parametric statistical tests. Linear mixed effects regression (LMER) models were fitted using the 'lme4' (Bates, 2013) package in R to investigate the effect of ACTH and metyrapone hormone administration (compared to saline controls) on the CORT response in gravid and non-gravid females after 3 h of capture-restraint. Log-transformed CORT was the response variable. Predictors were group (gravid female, non-gravid female), time, and treatment as fixed main effects (with interaction terms included for all). Tuatara ID was included as a random effect to account for repeat sampling of individuals for baseline and stress-response CORT values. The 'languageR' package (Baayen, 2011) was used to compute *P*-values based on Markov-chain Monte Carlo (MCMC) sampling. Significance for all tests was assumed at $p < 0.05$.

Results

Unexpectedly, ACTH and metyrapone treatments did not significantly influence the CORT stress response in gravid and non-gravid females compared with saline controls (Table A.1), though the trend of the CORT response for both treatments (in relation to saline controls) was as predicted (Fig A.1). I did not observe a significant effect of treatment on oviposition. During the 5-day holding period, one female from the ACTH group produced two eggs and one female from the saline group produced one egg, with no females from the metyrapone group producing eggs. Furthermore, a significant CORT response to 3 h capture-restraint and treatment was observed in non-gravid females (Table A.1b), but not in gravid females (Table A.1).

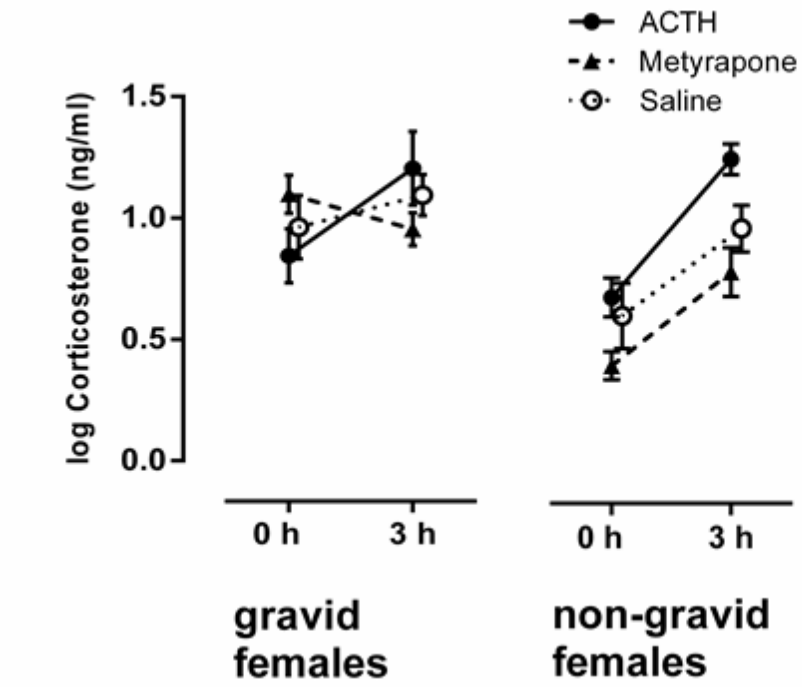

Figure A.1: CORT response to 3 h capture-restraint following either an ACTH challenge (solid line w/ closed circles), metyrapone challenge (dashed line w/ triangles) or saline control (dotted line w/ open circles) in gravid and non-gravid female tuatara (Sphenodon punctatus). Data points represent mean ± SE log CORT concentration (ng/ml) of baseline (0 h) and stress response (3 h) samples. No significant difference was found between treatment and saline controls for either group ($P>0.05$).

Table A.1: Results from linear mixed effects regression models explaining variation in measures of baseline and stress response CORT secretion (ng/ml) as a function of time (0-3 h) after explaining variation accounted for by the fixed effects of hormone treatment (ACTH, metyrapone, saline), capture-restraint of 3 h and the interaction of these effects for a) gravid females and b) non-gravid females.

Fixed effect predictor	**Estimate**	**Lower 95%CI**	**Upper 95%CI**	***P***
c) gravid females				
saline (intercept)	0.962	0.108	8.871	<0.001
ACTH	-0.118	0.153	-0.769	0.449
metyrapone	0.136	0.153	0.887	0.384
capture-restraint time (saline)	0.132	0.153	0.861	0.398
capture-restraint time x ACTH	0.228	0.217	1.051	0.304
capture-restraint time x metyrapone	-0.276	0.217	-1.273	0.215
d) non-gravid females				
saline (intercept)	0.575	0.092	6.238	<0.001
ACTH	0.073	0.124	0.590	0.561
metyrapone	-0.197	0.124	-1.593	0.125
capture-restraint time (saline)	0.345	0.110	3.127	0.005
capture-restraint time x ACTH	0.201	0.148	1.135	0.188
capture-restraint time x metyrapone	0.025	0.148	0.169	0.867

Coefficient estimates (positive or negative) are shown and indicate direction of the linear regression from the specified intercept. 95% credible intervals (CI) are shown. P-values based on MCMC sampling are shown and statistically significant results are indicated in bold. A random intercept term for individual was included in the model.

Discussion

In an attempt to elucidate the underlying causes of the dampened CORT response in gravid female tuatara, I administered an ACTH challenge to determine if gravid females are operating at maximal CORT secretion during the nesting life-history stage. I also used ACTH and metyrapone treatments to explore the hypothesis that modulation of CORT secretion influences the timing of oviposition. Unexpectedly, I

did not observe a significant effect from either treatment compared to saline controls in gravid or non-gravid females. However, I did observe a response to 3 h capture-restraint in the saline groups that is consistent with my 24 h capture-restraint results, namely that gravid females had a dampened stress response compared to non-gravid females.

It is surprising that plasma CORT concentrations were unaffected by both ACTH and metyrapone hormone challenges, as studies on other reptile species have seen marked responses from similar doses in shorter time-frames than in this study (Cartledge and Jones, 2007; Klukowski, 2011; Preest *et al.*, 2005; Romero and Wikelski, 2006). A possible explanation could be that the timing of the CORT response sample at 3 h was either too soon or too late to detect a significant treatment effect. Lack of significant effect from hormonal treatment at certain sample times was observed in related studies; for example, ACTH challenges produced plasma CORT concentrations that were significantly higher than saline controls at 1 h and 6 h only in male alligators (*Alligator mississippiensis*) (Mahmoud *et al.*, 1996) and in New Zealand common geckos at 1 h only (Preest *et al.*, 2005), but not at any other sample time points tested in either study. Another possibility explaining the absence of a significant treatment effect could be that my calculated hormone dosages may have been too low for my model species. Tuatara are a temperate climate ectotherm (with nocturnal T_b largely tracking ambient temperature), and I planned hormone dosage based on other reptiles that may experience warmer temperatures and therefore may have different rates of hormone metabolism. My preliminary results will be useful as a starting point to inform potential studies investigating mechanisms of CORT modulation in tuatara during nesting. Due to permitting constraints and research timelines, I was unable to carry out a pilot study beforehand. Therefore, it would be beneficial to investigate the dose-dependent response to both ACTH and metyrapone treatment over a time-series to inform future research and to understand mechanisms and importance of CORT secretion in tuatara when challenged with exogenous hormones.

In the present study, I did not observe an influence of ACTH or metyrapone treatment on oviposition, but as plasma CORT concentrations were not significantly

affected by treatments in this study, these results do not illuminate my hypothesis. The functional significance of elevated baseline CORT in gravid oviparous reptiles (and the potential relationship with oviposition) has received little attention, even though several oviparous reptile species exhibit elevated concentrations of baseline CORT concentrations directly preceding oviposition (Cree and Tyrrell, 2001; Jessop, 2001; Moore and Jessop, 2003). Female tuatara have a unique egg development/maintenance strategy, retaining their eggs for the longest known period in reptiles (7-9 months) (Cree *et al.*, 1992). Therefore, elevated baseline CORT concentrations during the nesting life-history stage in tuatara cannot be attributed to sexual receptivity or ovulation, as these mating events are greatly separated in time from nesting. A prior study on tuatara shows that baseline CORT concentrations decline shortly after oviposition (Cree and Tyrrell, 2001) , which suggests a possible role in the timing of egg-laying, and other studies suggest hormonal control of ovulation and parturition in viviparous and oviparous reptiles (Jones and Guillette, 1982). For example, it was shown that embryonic production of CORT may trigger parturition in viviparous southern snow skinks (*Niveoscincus microlepidotus*) (Girling and Jones, 2006) and exogenous elevation of baseline CORT concentrations in gravid eastern three-lined skinks (*Bassiana duperreyi*) induced 'premature' oviposition (Radder *et al.*, 2008). These findings suggest that up-regulating baseline CORT secretion during nesting could function to stimulate oviposition in gravid reptiles.

Conclusions

I provide results from the first hormone manipulation studies investigating modulation of CORT secretion in tuatara. While the present study provides baseline information for future hormone manipulation studies in tuatara, further studies will be necessary. It would be useful to carry out a dose-response study for ACTH and metyrapone administration with several response sampling times in order to determine suitable dosages and durations to observe significant effects. This would inform future studies investigating whether CORT is being secreted at maximal levels during nesting and if/how it plays a role in oviposition and nesting behaviour in tuatara, the sole extant representative of an ancient order of reptiles.

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