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Geographic variation in thermal physiological traits: the role of thermal stress on telomere length in a polymorphic ectotherm

Daniel Ritchie

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Geographic variation in thermal physiological traits: the role of thermal stress on telomere length in a polymorphic ectotherm

Abstract

Geographic variation in thermal adaption is common across ectothermic organisms due to their intrinsic metabolic link with ambient temperatures. However, in the face of altered thermal regimes, ectotherms may incur an array of biomolecular costs associated with suboptimal temperatures. As such, this thesis aimed to investigate the effect of altered thermal regimes on the thermal physiology of two populations of a polymorphic lizard Ctenophorus pictus. Lizards from both a warm adapted and cool adapted population were acclimated in either a warm or cool treatment for three-months, and various metrics of thermal physiology were assessed pre-and post-acclimation. Chapter 2: Populations were found to differ significantly in several traits such as metabolic rate, body size, basking behaviour and reproductive investment, potentially indicating thermal adaption between the populations. Surprisingly, temperature treatment only influenced post-maturity skeletal growth, with individuals in the cool treatment growing considerably longer, suggesting equivalent levels of phenotypic plasticity across both populations. Chapter 3: Telomere length as a potential biomolecular cost of sub-optimal temperatures was quantified via qPCR from blood samples in the field, pre-and post-acclimation. Surprisingly, telomere length was only significantly shorter for the cold-adapted population in the cool room, contrasting with the majority of theoretical predictions. Increased basking activity due to the decreased temperatures may account for such a result; however, no such trend was seen in the warm adapted population. Population-specific telomere response to altered thermal regimes found in this experiment is the first of its kind and represents a tantalizing new ecological research area. Overall, the results of this thesis demonstrate levels of thermal adaptation within both populations of C. pictus as well as levels of thermal plasticity to altered thermal regimes. The potential telomeric cost of thermal acclimation in ectotherms is an underexplored phenomenon, with more research needed to delineate the complex and counterintuitive relationship between temperature and telomere response.

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Christopher Friesen

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Daniel Ritchie

March 2021

A thesis submitted in part fulfilment of the requirements of the Honours degree of Bachelor
of Conservation Biology (Hons) in the School of Earth, Atmospheric and Life Sciences
University of Wollongong 2021

Statement of originality

This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the degree of BCons Biol Hons. It does not contain any material published by another person without due reference within the text. The field and laboratory work presented in this thesis was performed by the author, except where acknowledged. This thesis has not been submitted in part, or otherwise, for any other degree or qualification at any other university.

Daniel J. Ritchie

March 2021

Thesis Abstract

Geographic variation in thermal adaption is common across ectothermic organisms due to their intrinsic metabolic link with ambient temperatures. However, in the face of altered thermal regimes, ectotherms may incur an array of biomolecular costs associated with sub-optimal temperatures. As such, this thesis aimed to investigate the effect of altered thermal regimes on the thermal physiology of two populations of a polymorphic lizard *Ctenophorus pictus*. Lizards from both a warm adapted and cool adapted population were acclimated in either a warm or cool treatment for three-months, and various metrics of thermal physiology were assessed pre-and post-acclimation. **Chapter 2**: Populations were found to differ significantly in several traits such as metabolic rate, body size, basking behaviour and reproductive investment, potentially indicating thermal adaption between the populations. Surprisingly, temperature treatment only influenced post-maturity skeletal growth, with individuals in the cool treatment growing considerably longer, suggesting equivalent levels of phenotypic plasticity across both populations. **Chapter 3**: Telomere length as a potential biomolecular cost of sub-optimal temperatures was quantified via qPCR from blood samples in the field, pre-and post-acclimation. Surprisingly, telomere length was only significantly shorter for the cold-adapted population in the cool room, contrasting with the majority of theoretical predictions. Increased basking activity due to the decreased temperatures may account for such a result; however, no such trend was seen in the warm adapted population. Population-specific telomere response to altered thermal regimes found in this experiment is the first of its kind and represents a tantalizing new ecological research area. Overall, the results of this thesis demonstrate levels of thermal adaptation within both populations of *C. pictus* as well as levels of thermal plasticity to altered thermal regimes. The potential telomeric cost of thermal acclimation in ectotherms is an underexplored phenomenon, with more research needed to delineate the complex and counterintuitive relationship between temperature and telomere response.

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Finally, I would also like the thank the fantastic lizards involved in this study, although I did not meet you, it has been an honour and a privilege to study you, and I hope I meet some of your friends one day.

Abbreviations

ATP- Adenosine 5'-triphosphate

BCI – Body condition index

CTmax – Maximum critical temperature

CTmin – Minimum critical temperature

ETC – Electron transport chain

GSI – Gonadal somatic index

HSP – Heat shock proteins

POL – Pace of life

REDOX – Oxidation-reduction reactions

ROS – Reactive oxygen species

rTL – Relative telomere length

SVL – Snout vent length

Topt – Thermal optimum temperature

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Chapter 1:

Review of thermal effects on oxidative stress in vertebrate ectotherms.

Abstract

Human-induced climate change is occurring rapidly. Ectothermic organisms are particularly vulnerable to these temperature changes due to their reliance on environmental temperature. The extent of ectothermic thermal adaptation and plasticity in the literature is well documented; however, the role of oxidative stress in these processes requires significant attention. Oxidative stress occurs when reactive oxygen species, generated through aerobic respiration, overwhelm antioxidant defences and damage crucial biomolecules. Flow on effects of oxidative damage include the alteration of life-history traits and reductions in whole-organism fitness. Here I review the literature addressing temperature effects on oxidative stress in ectotherms. Acute and acclimation temperature treatments produce distinctly different results and highlight the role of phylogeny and thermal adaptation in shaping oxidative stress responses. Acute treatments on organisms adapted to stable environments always produced significant oxidative stress responses, whilst organisms adapted to variable conditions had elevated capacity to deal with temperature changes and mitigated oxidative stress. In acclimation treatments, the warmer treatment tended to produce significantly less oxidative stress than cooler treatments in reptiles, whilst in some eurythermal fish species, no oxidative stress response was observed. These results highlight temperature dependant oxidative stress responses and the importance of phylogeny to this relationship. I conclude with recommendations on experimental procedures to investigate these phenomena with particular reference to thermal plasticity, adaptation and biogeographic variation that provide the most significant benefits to adaptable populations. These results have potential conservation ramifications as they may be able to shed light on the physiological effects of temperature increase in some ectotherms.

Introduction

Global climate is currently experiencing unprecedented rates of change due to the excessive release of greenhouse gases (Diffenbaugh and Field 2013). Large scale warming of the planet is predicted, with an increase of 4 degrees by the next century (IPCC, 2014). Already, temperature changes are causing significant negative impacts upon ecosystems and organisms (Parmesan 2006). For example, changes in temperature impact species distributions (VanDerWal *et al.* 2013), alter trophic structure (Petchey *et al.* 1999), and change life histories (Peck *et al.* 2006). These phenomena occur by altering the physiology of organisms though heat-stress responses, these responses happen when an organism experiences ambient heat outside its thermal limits, imposing various metabolic costs (Kingsolver *et al.* 2013). Ectotherms are predicted to be the most at risk in this climate crisis due to their inability to use metabolic heat to maintain body temperature, making them vulnerable to temperature fluctuations (Sheridan and Bickford 2011; Seebacher *et al.* 2015). A known cost of these temperature fluctuations is oxidative stress (Speakman *et al.* 2015).

Various empirical studies investigating long-term thermal acclimation in ectotherms uncover non-linear oxidative stress responses to temperature (Ballen *et al.* 2012; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Dupoue *et al.* 2020). To review these effects, I outline the generation of reactive oxygen species (ROS) and how organisms can prevent oxidative stress. Furthermore, I outline some critical life-history traits that control, and are altered by oxidative stress before investigating the experimental literature on temperature and oxidative stress in ectotherms. Finally, I review potential mechanisms controlling these phenomena and propose experimental procedures to investigate these effects in thermal adaptation plasticity and biogeographic variation.

Generation of reactive oxygen molecules

Many organisms produce most of their energy via aerobic respiration—a process that is also the primary producer of damaging ROS (Dowling and Simmons 2009). ROS are reactive molecules and free radicals derived from molecular oxygen, which are the by-products of oxidation-reduction reactions (REDOX) (Dowling and Simmons 2009). The mitochondria are the primary source of cellular energy in the form of ATP via oxidative phosphorylation (Brand 2016), and also the chief source of ROS within the cell (Dowling and

Simmons 2009). Oxidative phosphorylation is a process that requires several enzymatic complexes embedded in the inner mitochondrial membrane, termed the electron transport chain (ETC) (Andreyev *et al.* 2005). Electrons are transported through the ETC proteins until they reach molecular oxygen, aiding the movement of protons (H^+) into the inner membrane space of the mitochondria. The energy is stored as a proton gradient and utilised to drive ATP synthase, which catalyses the production of ATP from ADP and P_I (phosphate). During this process, a fraction of the oxygen molecules, usually converted into water, are converted into a variety of ROS (Andreyev *et al.* 2005). A singular electron reduction produces a superoxide anion, which becomes the progenitor for many ROS. The most common ROS made in this process are the superoxide anion (O_2^-) as well as hydrogen peroxide (H_2O_2) plus subsequent molecules such as the hydroxyl anion and radical (OH^-, •OH, respectively) (Halliwell 1991). ROS production also occurs via other REDOX reactions within an organism, such as immune-defensive responses to pathogens (Apel and Hirt 2004).

ROS is the Dr Jekyll and Mr Hyde of the cell, playing both pro- and antagonistic roles. ROS facilitate a wide range of crucial biological processes such as; immune defence (Apel and Hirt 2004), as a signal/cue during growth and development (Covarrubias *et al.* 2008), cell apoptosis (Van Breusegem and Dat 2006) and modulation of stress responses (Gechev *et al.* 2006). However, when in large concentrations, ROS damage cellular function via DNA damage, lipid peroxidation, and protein damage (Ballard and Melvin 2010). Cellular damage leads to a decrease in organism fitness, i.e., oxidative stress (Smith et al. 2016). ROS-scavenging molecules have evolved into an array of enzymatic and non-enzymatic antioxidants working in concert to quench ROS before it can impair cellular function (Costantini and Verhulst 2009). Superoxide dismutase (SOD) is a primary enzymatic antioxidant defence mechanism that catalyses superoxide's conversion to the less reactive hydrogen peroxide molecule (Andreyev *et al.* 2005). The enzyme Catalase (CAT) catalyses the decomposition of H_2O_2 into O_2 and H_2O. Another important enzymatic antioxidant is Glutathione (GSH), which has a variety of functions in both its enzymatic and reduced form (Andreyev *et al.* 2005). GSH's role is to scavenge superoxide and hydroxyl radicals and serve as an electron donor to several other enzymatic reactions in ROS detoxification (Hiraishi *et al.* 1991). Antioxidants can also be attained through diet; vitamin C, vitamin E, carotenoids, and polyphenols all help quench excess ROS (Bouayed and Bohn 2010). Additionally, cellular repair mechanisms, such as the proteasome, are continually operating to limit, repair or replace damage caused by ROS (Selman *et al.* 2002). The result is a hypothetically balanced system in which ROS are constantly produced and quenched; the level of this balance is the Oxidative Status. When antioxidants cannot quench ROS, the oxidative damage of biomolecules occurs and, subsequently, reduces organismal fitness (Metcalfe and Alonso-Alvarez 2010).

Increased temperature and Oxidative stress' influence on life-history

Life-history traits and the timing and duration of phenology and therefore individual or population level fitness, can be altered by increases in temperature and oxidative stress (Dowling and Simmons 2009; Forrest and Miller-Rushing 2010). Life-history traits are traits of an organism's life that impact population growth rates, such as life span, growth, fecundity, age at first reproduction and clutch size (Stearns 1982). For this review, I only briefly discuss a few major life-history traits and their relationship with oxidative stress (for full reviews see; Dowling and Simmons 2009; Monaghan *et al.* 2009; Metcalfe and Alonso-Alvarez 2010; Speakman *et al.* 2015). The basis for understanding life-history traits revolves around fitness trade-offs, which occur when organisms are forced to allocate finite resources among key life-history aspects such as reproduction or growth (Speakman *et al.* 2015).

Depending on oxidative balance, mitigating oxidative stress may influence life-history trade-offs through energy allocation to antioxidant defences (Monaghan *et al.* 2009). Processes such as growth, bodily maintenance and reproduction require significant amounts of energy and can leave cellular mechanisms vulnerable to ROS due to decreased antioxidants (Dowling and Simmons 2009). Interestingly, energetically costly life-history traits also require significant oxygen consumption increases, a factor traditionally associated with increased oxidative damage (Harman 1968). However, current theoretical predictions suggest that ROS production may be altered independently from oxygen consumption in some cases (Costantini 2019). Empirically, negative correlations between metabolic rate and oxidative stress are evident in fish (Salin *et al.* 2015b; Salin *et al.* 2018), reptiles and mammals (Speakman *et al.* 2004). A hypothesised reason for this is that elevated metabolism can result in higher levels of mitochondrial uncoupling, which is known to decrease ROS production (Bonawitz *et al.* 2007; Koch *et al.* 2021). Thus, generally, oxidative stress can impact life-history traits through trade-offs, either through energy allocation or increased oxygen consumption.

Growth, longevity and senescence are crucial components of life history and can shape population structure (Monaghan *et al.* 2009). Increased temperature is known to increase ectothermic growth (Sheridan and Bickford 2011). This increased growth can lead to increases in oxidative stress attributed causally to elevated mitochondrial activity (Furtado-Filho *et al.* 2007; De Block and Stoks 2008; Smith *et al.* 2016). Additionally, oxidative stress is a significant factor in cellular and organismal senescence and, subsequently, longevity in various organisms (Larsen 1993; de Castro *et al.* 2004; Smith *et al.* 2016; Marasco *et al.* 2017). The effects of growth and metabolic function on life history can be viewed under the metabolic pace of life theory, which is formed from the observation that organisms that live for relatively short periods have increased metabolism rates compared to organism that live for extended periods (Glazier 2015). Thus, oxidative stress provides a tantalizing link between metabolism and populations structure though impacts on longevity and the development of antioxidant defences (Speakman *et al.* 2015).

Fecundity is another life-history trait in which trade-offs occur between the investment in current reproduction, future reproduction and survival (Reznick 1985; Costantini 2018) . Oxidative stress is a critical factor in this trade-off. Increased reproductive effort (metabolic expenditure) correlates with increased susceptibility to oxidative damage (Alonso-Alvarez *et al.* 2004; Guerra *et al.* 2012; Olsson *et al.* 2012a; Plantamp *et al.* 2016). Although oxidative stress may occur with reproduction, species have adapted to minimise this. For example, the circulation of a yolk precursor vitellogenin, found in female painted dragon lizards, acts as an antioxidant to quench increased ROS production associated with reproductive effort (Lindsay *et al.* 2020). Therefore, in some species, females can have lower than predicted oxidative stress due to highly effective antioxidant mechanisms despite substantial metabolic investment (Borrás *et al.* 2003). However, as temperatures increases, the oxidative costs of reproduction may increase (Alonso-Alvarez *et al.* 2004). At higher temperature, increased oxidative stress may occur through elevated metabolic function generating ROS or reducing efficiency of antioxidants at higher temperatures (Bae *et al.* 2016). Thus, oxidative stress plays a significant role in influencing organisms' life-history traits; as temperatures increase, the delicate oxidative balance may be shifted and place organisms in situations where increased oxidative damage is unavoidable. Therefore, understanding ectothermic thermal biology is crucial in predicting the effects of long-term temperature change on populations' oxidative status.

Thermal biology of ectotherms and potential oxidative stressors

Temperature changes alter physiological processes (Kingsolver *et al.* 2013), and can increase oxidative stress (Birnie-Gauvin *et al.* 2017). This fact is particularly crucial for ectotherms, which are a polyphyletic group of organisms united by their inability to raise their body temperature through metabolism but do so via seeking out warm environments (Sheridan and Bickford 2011; Seebacher *et al.* 2015). Ectotherms' metabolic rates correlate substantially with ambient temperature (Pough 1980), making them physiologically sensitive to increases in temperature (Sheridan and Bickford 2011; Seebacher *et al.* 2015). Therefore, ectotherms have evolved a host of thermal adaptations, selecting genotypes that optimise bodily function matched to the expected environment via physiological and behavioural means (Angilletta 2009). Conversely, ectotherms may have strong phenotypically plastic responses to survive in an array of variable thermal environments (Lande 2015). The critical physiological benefit of deriving body temperature from the environment is that individuals can invest more energy in growth and reproduction per unit of food than endotherms (Pough 1980). As a trade-off for this benefit, ectotherms sacrifice performance breadth of proteins and cellular processes, with proteins needing to function over a wide range of temperatures (Angilletta 2009). Thus, protein performance and indirectly organism performance in ectotherms exhibit thermal optima (Martin and Huey 2008) (*Figure 1)*

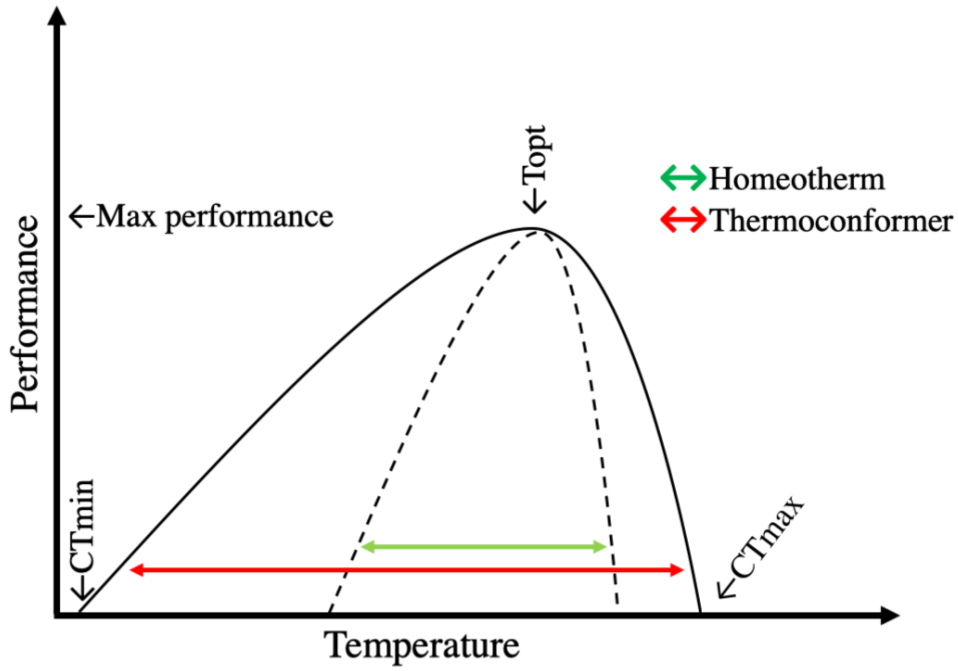


Figure 1*. Thermal performance curves of two hypothetical ectotherms, a homeotherm (dashed line) and a thermoconformer (solid line), from stable and variable thermal environments, respectively. Demonstrating Topt (ideal performance temperature), CTmax (upper thermal limit), CTmin (lower thermal limit) and operative performance breadths.*

The thermal optimum (Topt) is defined as the ideal temperature for physiological processes (performance) and can be a characteristic of a population or species. Whilst critical temperatures (CT), both high, CTmax, and low, CTmin, are defined at the point at which the organisms transition to anaerobic mitochondrial respiration (*Figure 1*) (Pörtner 2002a). As temperature increases, performance increases toward CTmax; at this point, some ectotherms can behaviourally modulate their body temperature and to maintain Topt (Pörtner 2002a). Behavioural thermoregulation may relax selection on temperature-sensitive physiological traits, a phenomena called “The Bogert effect” (see Figure 1. homeotherm performance curve Bodensteiner *et al.* 2020). Effective thermoregulation in ectotherms may limit the evolution of physiological ability to cope with warming temperatures and then experience significant oxidative stress when behavioural means can no longer limit heat exposure (Bodensteiner *et al.* 2020). A variety of empirical studies on or of ectothermic species that experimentally alter temperature in laboratory conditions find that increased temperature does not necessarily lead to increased oxidative stress, and this may be due to wide thermal performance breadth generated by adaptations to variable environments and plasticity (Ballen *et al.* 2012; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Dupoue *et al.* 2020). This insensitivity to long-term warming may be either an artefact of experimental design flaws or an ectothermic thermal tolerance mechanism to oxidative stress (see Candidate mechanisms to explain acute and acclimation responses. Sections pg. 30). Potential experimental design flaws may occur through poor selection of biomolecular markers to quantify oxidative stress based on their functional roles within the oxidative system and the significance of tissue types used.

Biomolecular markers of oxidative stress

A variety of biomolecular markers and tissues can be used to quantify oxidative stress (full review of practices see Monaghan *et al.* 2009; Birnie-Gauvin *et al.* 2017). Oxidative stress markers fall into three categories, general ROS products, biomolecular markers of oxidative damage and antioxidants. Typically, studies assay the activity of these markers with commercial kits. However, all biomarkers measure different aspects of oxidative stress and depend on the tissues measured, and therefore selection of an array of biomarkers will impact experimental validity, outcomes and interpretation (Monaghan *et al.* 2009).

Markers that measure ROS related by-products within the sample, such as dROMS, do not measure oxidative stress *per se,* rather an indicator of ROS generation levels (Monaghan *et al.* 2009; Hõrak and Cohen 2010). Biomarkers of oxidative damage such as Malondialdehyde (MDA), 8-oxo-dg DNA damage (OXO) and protein carbonyls can be indicative of reduced fitness, however only via understanding the levels of cellular damage and the potential physiological impacts can provide the link to whole organismal fitness (Ho *et al.* 2013). Therefore, making these links is methodologically and logistically challenging (Hõrak and Cohen 2010). Measuring an organism's antioxidant capacity, as opposed to ROS damage, is useful as it demonstrates the organism's ability to quench ROS and therefore limit oxidative damage (Catoni *et al.* 2008). However, antioxidants alone are insufficient at measuring oxidative stress, as their levels are not always relative to ROS production (Hõrak and Cohen 2010). Antioxidants can be either endogenous, produced within the organism, or exogenous, gained through diet (Bouayed and Bohn 2010). Each antioxidant has a high specificity to particular ROS species (Surai *et al.* 1996) and have varying degrees of biological relevance to fitness. One key factor of oxidative damage that can be measured is the organisms' ability to repair oxidative damage (Selman *et al.* 2002). Implementation of metrics such as the proteasome, a group of protein complexes that degrade damaged proteins, will significantly help understand the biological relevance of oxidative stress (Keller *et al.*

2000; Selman *et al.* 2002). Therefore, a wide range of oxidative stress markers is key to understanding an organism's whole oxidative status at a given time (Monaghan *et al.* 2009).

Significant variation occurs among the tissues of an individual in the concentration of oxidative status markers (Madeira *et al.* 2016). When specific tissues are damaged, different aspects of fitness are impacted depending on the tissue's function. For example, oxidative damage to the liver can limit the organism's ability to detoxify the blood and store glycogen (Oettl *et al.* 2013), and oxidative damage to the brain may limit cognitive function, affecting behaviour and response times (Forster *et al.* 1996). Respiratory surfaces such as lungs and gills accumulate high concentrations of ROS-related products with temperature stress, whilst brain tissues can show minimal to no change within the same organism (Madeira *et al.* 2013; Madeira *et al.* 2016). Thermal sensitivity of tissues is essential to consider when measuring the effects of temperature stress, for example, brains will experience minimal temperature fluctuation compared to muscle tissue (Kiyatkin 2010). However, in studies of endangered or threatened species, terminal sampling is often unacceptable. Thus, samples of peripheral blood may provide adequate information on oxidative status whilst also enabling researchers to perform repeated measures (Hõrak and Cohen 2010).

As markers and tissues generally differ in their biological origin and function, levels of measured OS within individuals may not be correlated. Thus, understanding the question of research interest must be paramount in guiding the choice of markers and tissues to study. Pilot studies are needed to test the correlations between biomolecular markers and between tissues in the same or closely related species. However, if such studies do not exist or are not feasible, a range of biomolecular markers should provide useful information. At a minimum, one measure of oxidative damage and antioxidant capacity will be adequate to assess the individual's oxidative status in question, but preferably up to three of each will achieve more in-depth interpretations (Hõrak and Cohen 2010). Although lacking some specificity, peripheral blood samples give a general view of whole organismal oxidative stress and allow for repeated measurements providing more statistical power than cross-sectional approaches (Hõrak and Cohen 2010).

Telomeres are a biomarker of oxidative stress, that link to cellular senescence and longevity, a key life-history trait (Monaghan 2014). The majority of research conducted on telomeres and telomerase dynamics has been conducted on endothermic organisms, particularly humans, birds, and laboratory breed mammals (Shammas 2011; Maciejowski and de Lange 2017; Muñoz-Lorente *et al.* 2019). Telomeres are tandem non-coding nucleotide repeats at the end of chromosomes, which ensure when linear DNA replicates, coding DNA is conserved (Kawanishi and Oikawa 2004). As telomeres shorten with each replication, they provide a limit on how many times a cell can divide and thus may correlate with growth rates (Monaghan and Ozanne 2018). Telomeres respond to stress and aging by lengthening or shortening (Von Zglinicki 2002; Heidinger *et al.* 2012; Olsson *et al.* 2018a). The telomere length maintenance process occurs through the enzyme telomerase, which can increase the limit of cell replication significantly (Chan and Blackburn 2004). Shortened telomeres can instigate cellular senescence and are linked to various age-related diseases in humans (Barnes *et al.* 2019). Conversely, overly long telomeres, brought about by high telomerase expression, can increase cancer formation rates in certain taxa (McNally *et al.* 2019). Thus, stabilizing selection may operate to keep telomeres at an intermediate-length to mitigate the effects of either short or long telomeres (Murnane 2012). Additionally, telomeres have been linked to costs of reproductive investment (Sudyka 2019) and influence many life history trade-offs (Young 2018; Casagrande and Hau 2019; Whittemore *et al.* 2019). Telomeres are a biomarker of oxidative stress as increased ROS can increase telomere attrition via ROS attacking the guanine-rich telomeric sequence, providing a critical link between oxidative stress and life-history traits (Houben *et al.* 2008; Chatelain *et al.* 2020).

Telomere dynamics (lengthening and shortening) is highly variable across taxa, populations, sexes, among and within individuals (Olsson *et al.* 2018a). Thus, understanding the role of temperature in mediating ectothermic telomere responses is difficult (Olsson *et al.* 2018a). Generally, it is predicted that via increasing temperature, growth will increase, thus increasing cell proliferation and ROS production and telomere attrition (Chatelain *et al.* 2020). In the Painted Dragon lizard, negative correlations between growth and telomere length are observed (Friesen *et al.* 2021). Increased temperatures and, subsequently, growth rates in salmon have increased telomere attrition (McLennan *et al.* 2016). In zebra fish, treatment temperature was negatively correlated with telomere length (Rollings *et al.* 2014). However, in brown trout and nine-spined sticklebacks, increased temperature and growth rates had no impact on telomere attrition (Näslund *et al.* 2015; Noreikiene *et al.* 2017). In reptiles, only a few studies have experimentally investigated the effects of thermal acclimation on telomere dynamics. One such study, spotted snow-skinks (*Niveoscincus* *ocellatus)* were acclimated for 90 days at hot and cold temperatures and increased telomere erosion at hot temperatures occurred (Fitzpatrick *et al.* 2019). Similarly, in a desert agama (*Phrynocephalus przewalskii*) an experimental heat wave decreased telomere length significantly (Zhang *et al.* 2018). Unfortunately, the paucity of work on ectotherms in terms of telomere dynamics leaves many questions still to be addressed. This fact is particularly unfortunate because cellular replication and growth in ectotherms is more plastic in response to environmental drivers (especially temperature) than birds and mammals (Olsson *et al.* 2018a). This feature of ectothermic metabolism (and potentially oxidative stress), being tightly linked with temperature, yields the opportunity to segregate the costs of growth, development and age effects on telomere shortening.

Oxidative stress, temperature and life-history are intertwined with the thermal biology in ectotherms. Researchers now have the tools to quantify significant parts of an organism's oxidative status through various biomolecular markers. Recent empirical studies have found that long-term acclimation to temperatures above Topt can decrease oxidative stress levels in ectotherms, which is a finding in line with cellular investigations into the subject but unexpected in whole organism studies (Table 1). To understand these studies, I conduct a small-scale systematic review of empirical literature focused on experimental manipulation of temperature and measurement of oxidative stress to assess the variety of methodological and biological differences.

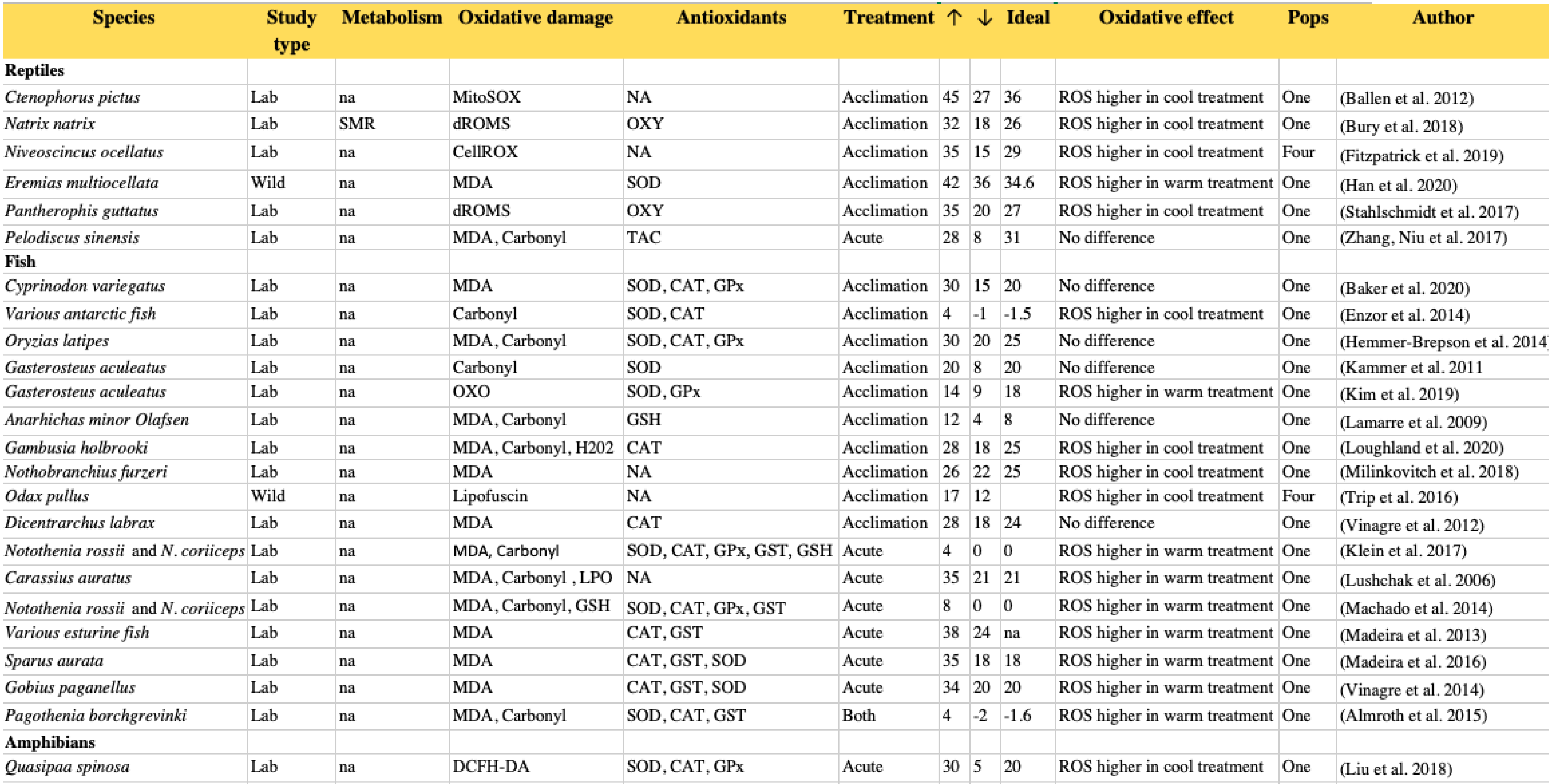
Systematic review methods

A systematic literature review using the database Web of Science was conducted focusing on studies that experimentally manipulate temperatures in ectothermic vertebrates and measured oxidative stress. A large amount of work has already been done on the mechanisms of oxidative stress in endotherms, particularly humans and mice (Jomova and Valko 2011; Rahman *et al.* 2012; Furukawa *et al.* 2017). Additionally, ectothermic thermal adaptations have been widely explored (Angilletta 2009), but the combination of both ectothermic thermal response and oxidative stress has received relatively little exploration (Monaghan *et al.* 2009). Thus, the combination of the key topic areas "oxidative stress", "oxidative damage", "temperature", and "ectothermic" were used in the search. The last search was conducted on the 28th of August 2020, and all articles were compiled into an Endnote document for screening. Papers were removed if they focused-on invertebrates as there is considerably less data available on oxidative stress and thermal adaptations for this clade, alongside uncertainty about differences in the underlying mechanisms that may control such responses (Abele *et al.* 2007). Additionally, papers were removed if they were in vitro, lacked a measure of oxidative stress, did not manipulate/measure temperature, or measured telomere attrition. Cell proliferation plays a central role in determining telomere attrition rates and thus would be inconsistent across study species (Monaghan and Ozanne 2018). A total of 63 papers were found, 29 were removed on invertebrates, and an additional 15 were removed for not measuring oxidative stress parameters. Through searching citations of key articles, another five papers were added to the list. Thus, 24 papers were included in this review.

Legend
-Metabolism= Metric used to quantify metabolic rate
-Study type= Conducted in either laboratory or wild
-Oxidative damage= metric used to quantify oxidative stress
-Antioxidant = metric used to quantify antioxidant capacity
-Treatment= Thermal treatment type

-↑= Highest temperature in the experiment
-↓= Lowest temperature in the experiment
-Ideal= Optimum temperature for the species
-Oxidative effect= effect of temperature on oxidative stress
-Pop= amount of populations used in the experiment

Table 1. *Empirical studies focusing on temperatures effect on oxidative stress in ectotherms, compiled from the database Web of science conducted on 28th of August 2020.*



Species	Study type	Metabolism	Oxidative damage	Antioxidants	Treatment	↑	↓	Ideal	Oxidative effect	Pops	Author
Reptiles											
Ctenophorus pictus	Lab	na	MitoSOX	NA	Acclimation	45	27	36	ROS higher in cool treatment	One	(Ballen et al. 2012)
Natrix natrix	Lab	SMR	dROMS	OXY	Acclimation	32	18	26	ROS higher in cool treatment	One	(Bury et al. 2018)
Niveoscincus ocellatus	Lab	na	CellROX	NA	Acclimation	35	15	29	ROS higher in cool treatment	Four	(Fitzpatrick et al. 2019)
Eremias multiocellata	Wild	na	MDA	SOD	Acclimation	42	36	34.6	ROS higher in warm treatment	One	(Han et al. 2020)
Pantherophis guttatus	Lab	na	dROMS	OXY	Acclimation	35	20	27	ROS higher in cool treatment	One	(Stahlschmidt et al. 2017)
Pelodiscus sinensis	Lab	na	MDA, Carbonyl	TAC	Acute	28	8	31	No difference	One	(Zhang, Niu et al. 2017)
Fish											
Cyprinodon variegatus	Lab	na	MDA	SOD, CAT, GPx	Acclimation	30	15	20	No difference	One	(Baker et al. 2020)
Various antarctic fish	Lab	na	Carbonyl	SOD, CAT	Acclimation	4	-1	-1.5	ROS higher in cool treatment	One	(Enzor et al. 2014)
Oryzias latipes	Lab	na	MDA, Carbonyl	SOD, CAT, GPx	Acclimation	30	20	25	No difference	One	(Hemmer-Brepson et al. 2014
Gasterosteus aculeatus	Lab	na	Carbonyl	SOD	Acclimation	20	8	20	No difference	One	(Kammer et al. 2011
Gasterosteus aculeatus	Lab	na	OXO	SOD, GPx	Acclimation	14	9	18	ROS higher in warm treatment	One	(Kim et al. 2019)
Anarhichas minor Olafsen	Lab	na	MDA, Carbonyl	GSH	Acclimation	12	4	8	No difference	One	(Lamarre et al. 2009)
Gambusia holbrooki	Lab	na	MDA, Carbonyl, H202	CAT	Acclimation	28	18	25	ROS higher in cool treatment	One	(Loughland et al. 2020)
Nothobranchius furzeri	Lab	na	MDA	NA	Acclimation	26	22	25	ROS higher in cool treatment	One	(Milinkovitch et al. 2018)
Odax pullus	Wild	na	Lipofuscin	NA	Acclimation	17	12		ROS higher in cool treatment	Four	(Trip et al. 2016)
Dicentrarchus labrax	Lab	na	MDA	CAT	Acclimation	28	18	24	No difference	One	(Vinagre et al. 2012)
Notothenia rossii and *N. coriiceps*	Lab	na	MDA, Carbonyl	SOD, CAT, GPx, GST, GSH	Acute	4	0	0	ROS higher in warm treatment	One	(Klein et al. 2017)
Carassius auratus	Lab	na	MDA, Carbonyl , LPO	NA	Acute	35	21	21	ROS higher in warm treatment	One	(Lushchak et al. 2006)
Notothenia rossii and *N. coriiceps*	Lab	na	MDA, Carbonyl, GSH	SOD, CAT, GPx, GST	Acute	8	0	0	ROS higher in warm treatment	One	(Machado et al. 2014)
Various esturine fish	Lab	na	MDA	CAT, GST	Acute	38	24	na	ROS higher in warm treatment	One	(Madeira et al. 2013)
Sparus aurata	Lab	na	MDA	CAT, GST, SOD	Acute	35	18	18	ROS higher in warm treatment	One	(Madeira et al. 2016)
Gobius paganellus	Lab	na	MDA	CAT, GST, SOD	Acute	34	20	20	ROS higher in warm treatment	One	(Vinagre et al. 2014)
Pagothenia borchgrevinki	Lab	na	MDA, Carbonyl	SOD, CAT, GST	Both	4	-2	-1.6	ROS higher in warm treatment	One	(Almroth et al. 2015)
Amphibians											
Quasipaa spinosa	Lab	na	DCFH-DA	SOD, CAT, GPx	Acute	30	5	20	ROS higher in cool treatment	One	(Liu et al. 2018)

Review of empirical studies into temperatures effect on oxidative stress in ectotherms

Sample size of empirical studies in *Table 1* was relatively small, 24 in total, six (25%) on reptiles (Ballen *et al.* 2012; Stahlschmidt *et al.* 2017; Zhang *et al.* 2017; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Han *et al.* 2020), 17 (70%) on fish (Lushchak and Bagnyukova 2006; Lamarre *et al.* 2009; Kammer *et al.* 2011; Vinagre *et al.* 2012; Madeira *et al.* 2013; Enzor and Place 2014; Hemmer-Brepson *et al.* 2014; Machado *et al.* 2014; Vinagre *et al.* 2014; Almroth *et al.* 2015; Madeira *et al.* 2016; Trip *et al.* 2016; Kim *et al.* 2017; Klein *et al.* 2017; Milinkovitch *et al.* 2018; Baker *et al.* 2020; Loughland and Seebacher 2020) and one (5%) on amphibians (Liu *et al.* 2018). Therefore, the conclusions are limited. Generally, oxidative stress increased when temperatures treatments were further from the organism's ideal temperature. Nine (37.5%) studies found oxidative stress levels higher in the cooler treatment (Ballen *et al.* 2012; Enzor and Place 2014; Stahlschmidt *et al.* 2017; Bury *et al.* 2018; Liu *et al.* 2018; Milinkovitch *et al.* 2018; Fitzpatrick *et al.* 2019; Loughland and Seebacher 2020), nine (37.5%) in warmer treatments (Lushchak and Bagnyukova 2006; Madeira *et al.* 2013; Machado *et al.* 2014; Vinagre *et al.* 2014; Almroth *et al.* 2015; Klein *et al.* 2017; Kim *et al.* 2019; Han *et al.* 2020), and six (25%) found no significant difference (Lamarre *et al.* 2009; Kammer *et al.* 2011; Vinagre *et al.* 2012; Hemmer-Brepson *et al.* 2014; Zhang *et al.* 2017). Acclimation studies tended to find oxidative stress was most notable at the lower acclimation temperature. Acute treatments tended to elicit oxidative stress at higher temperatures; however, 78% of acute studies did not investigate the effects of cold (Machado *et al.* 2014; Vinagre *et al.* 2014; Almroth *et al.* 2015; Madeira *et al.* 2016; Klein *et al.* 2017; Zhang *et al.* 2017). The only studies demonstrating no oxidative stress response to temperature were in fishes (Lamarre *et al.* 2009; Kammer *et al.* 2011; Vinagre *et al.* 2012; Hemmer-Brepson *et al.* 2014; Baker *et al.* 2020), suggesting phylogenetic differences in oxidative stress responses. Only one study quantified metabolism in response to temperature treatments (Bury *et al.* 2018).

Acute treatments

Acute thermal treatments are sharp increases in ambient temperature. These temperature increases are generally large and aim to take the organism well outside of Topt. Sometimes, these studies are terminal, heating the organism until it reaches CTmax. Studies such as these aim to understand the thermal limitations of ectotherms and the physiological processes that fail, resulting in death at high or low temperatures (Vinagre *et al.* 2012). Thus, such experiments' results depend on the thermal evolutionary adjustments and plasticity the species possesses before the acute thermal challenge. In the process of heating an organism at a high rate, heat stress responses occur. Proteins denature, and a variety of heat shock proteins (HSP) activate to conserve bodily function by re-establishing correct protein shape and limiting protein aggregation (Angilletta 2009). Expression of HSP and other thermal stress responses can be energetically/metabolically costly and are predicted to produce excess ROS (Martindale and Holbrook 2002). All but one study (Liu *et al.* 2018) found that acute increases in temperatures resulted in increases in oxidative stress. This study used a high temperature that was much closer to Topt than the cooler treatment, potentially confounding the interpretation of results.

Of the twenty-four primary studies we found, nine used an acute thermal treatment (Lushchak and Bagnyukova 2006; Madeira *et al.* 2013; Machado *et al.* 2014; Vinagre *et al.* 2014; Almroth *et al.* 2015; Madeira *et al.* 2016; Klein *et al.* 2017; Zhang *et al.* 2017). Of these, seven were conducted using fish. In fish, all seven studies found increased acute temperature resulted in higher levels of oxidative stress markers (Lushchak and Bagnyukova 2006; Madeira *et al.* 2013; Vinagre *et al.* 2014; Almroth *et al.* 2015; Madeira *et al.* 2016; Klein *et al.* 2017). However, two studies investigated cold stress and oxidative stress (Zhang *et al.* 2017; Liu *et al.* 2018). We only found one study on acute thermal stress in reptiles and amphibians, respectively (Zhang *et al.* 2017; Liu *et al.* 2018). No significant change in oxidative stress from an acute cold treatment was found in a turtle (*Pelodiscus sinensis)* (Zhang *et al.* 2017). In a frog (*Quasipaa spinosa),* exposure to high and low acute temperature treatments found that oxidative stress increased sharply in the cool treatment (Liu *et al.* 2018). As both organisms are thermoconformers, their thermal adaptations to deal with sharp temperature increases are predicted to be much more developed than many of the stenothermic marine fish species, suggesting that a lack of responses to acute heat treatments would be expected (Padmini and Geetha 2009). However, the lack of studies in reptiles and amphibians demonstrates a significant knowledge gap that impedes further phylogenetic comparison.

Physiological responses to acute temperatures are linked to an organism's ability to adjust its aerobic capacity at the molecular level (Pörtner 2002a). Oxygen delivery capacity is adaptively set to meet maximal oxygen demands between the high and low average environmental temperatures (Pörtner 2002a). Studies that acutely place organisms outside of expected extreme environmental temperatures elicit a strong thermal and oxidative stress responses; however, this was not evident in the two studies on thermoconformers (Zhang *et al.* 2017; Liu *et al.* 2018). These results may be due to both studies not placing the study organisms very far from their historical extremes. Regardless, these results suggest ectothermic oxidative stress responses to acute temperatures is indicative of evolutionary exposure to environmental thermal regimes (Pamplona and Barja 2011). Thus, the studies placing organisms under acute thermal challenges provide useful information in predicting and monitoring how species can handle intense heatwaves or cold snaps, which are expected to become more frequent as climate change continues (McKechnie and Wolf 2010).

Acclimation treatments

Thermal treatments that acclimate organisms seek to keep an organism at a set mean temperature for a minimum of one week up to 2 years. Through this period, organisms can adjust their physiological processes such that their thermal optimum shifts to maximise fitness in new environments (Rohr *et al.* 2018) (*Figure 2*). These studies seek to test phenotypic plasticity, defined as the expression of different phenotypes over an organism's life and is an evolutionary response to maximise fitness in variable environments. (Stearns 1989).

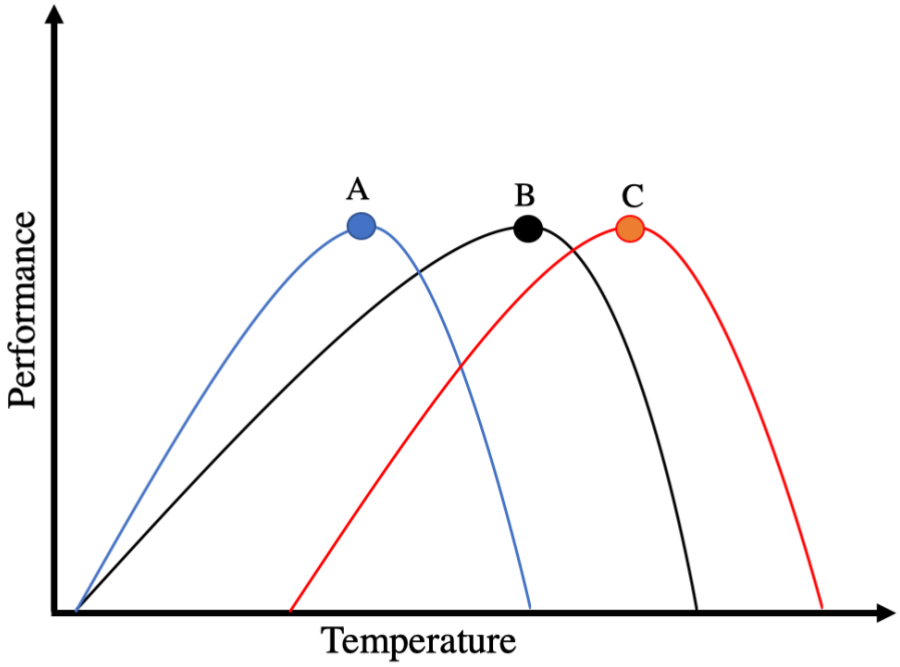


Figure 2. *Theoretical shifts in thermal optima following long term acclimation to low (**A**), optimal (**B**) and high (**C**) temperature in a hypothetical ectothermic species.*

Environmental temperature changes can happen over hours, days, weeks, seasons and years, depending on the environment (Huey and Kingsolver 1989). This variability means that ectotherms must adjust and/or adapt to temperature changes over a variety of temporal scales to optimise fitness (Angilletta 2009), which can lead to significant variation in acclimation capacity between individuals, populations and species (Loughland and Seebacher 2020). For example, an acclimation response to increased temperatures would be a physiological change in an organism that increases CTmax or increases thermal breadth (Rohr *et al.* 2018). The 'Bogert effect' suggests terrestrial ectotherms due to their effective behavioural modulation of bodily temperature, may lack scope for physiological adjustments to temperature compared to marine ectotherms. That is, behavioural thermoregulation allows terrestrial ectotherms to maintain tight control over their body temperature, which in turn allows selection to fine tune biochemical and physiological processes to a narrower range of temperatures (similar to endothermic birds or mammals) (Rohr *et al.* 2018). But unlike birds or mammals, ectotherms can only control temperature via behaviour within certain limits, unless they have the capacity to also acclimate to longer term changes in temperature mitigating some of the costs of finely tuning physiological functions to a particular temperature. Acclimation responses may have short-term oxidative stress costs via altering cellular membrane fluidity and increasing mitochondrial density (Kammer *et al.* 2011). Thus, the cost of acclimation can be measured as excess ROS production generated in a trade-off of acclimation (Loughland and Seebacher 2020). High levels of ROS in acclimating individuals is suggestive of inefficient acclimation and *vice versa*. By assessing acclimation capacity in this way, studies can make biologically relevant predictions about how species may be affected by long-term climatic warming. In the literature, 80% of studies found that cooler acclimation temperatures produced higher levels of oxidative stress markers (Ballen *et al.* 2012; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Dupoue *et al.* 2020). Higher environmental temperatures in ectotherms invariably increases metabolic rate (Pough 1980). These results suggest that ROS production might be altered independently from oxygen consumption during cold stress (Chung and Schulte 2020). Alternatively, enhanced metabolic capacity at high temperatures may increase the availability of resources for investment in antioxidants and cellular repair mechanisms (Dowling and Simmons 2009; Monaghan *et al.* 2009). Both of these theories contrast with the traditional notion of a strong correlation between oxygen consumption and oxidative stress (Harman 1968), suggesting further research on the drivers of oxidative stress in ectotherms is needed.

Of the twenty-four studies, sixteen were acclimation studies. Of those, five were on reptiles, and eleven were on fish. In reptiles, acclimatising treatments increased oxidative stress markers in cool treatments, despite assumed lower metabolic rate and retaining similar antioxidant capacity between treatments (Ballen *et al.* 2012; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Dupoue *et al.* 2020). The one exception to this trend was a study in which researches experimentally manipulated wild populations to experience increases in temperature and strong oxidative and immune-suppressive response was seen (Han *et al.* 2020). In acclimation studies on fish, four of eleven (36.6%) demonstrate that low temperatures increase oxidative stress markers (Enzor and Place 2014; Trip *et al.* 2016; Milinkovitch *et al.* 2018; Loughland and Seebacher 2020), five (45%) demonstrate no effect on oxidative stress markers in the heat treatments (Lamarre *et al.* 2009; Kammer *et al.* 2011; Vinagre *et al.* 2012; Hemmer-Brepson *et al.* 2014; Baker *et al.* 2020) and two (18.5%) show increased oxidative stress markers at high-temperature (Almroth *et al.* 2015; Kim *et al.* 2019). The role of thermal stress in mediating oxidative stress responses may be largely dependent on the study species' thermal biology, highlighting the role of the evolutionary history or adaptation and environmental stability guide these responses to acclimation treatments.

Terrestrial, benthic marine, and shallow aquatic organisms inhabit and have evolved in environments with vastly different thermal stability. Terrestrial environments can experience ten-fold differences in dial and seasonal temperatures depending on latitude (Sunday *et al.* 2014). Endotherms, such as birds and mammals, solve the problem of huge swings in temperature by making and storing the own heat with the aid of feathers, hair and subcutaneous fat (Porter and Kearney 2009). Terrestrial organisms such as reptiles, maintain thermal stability to some degree by behaviourally modulating their temperature, so much so that even in laboratory conditions, they may be able to mask biological variation in physiological adaptation (Taylor *et al.* 2020). In contrast, water limits the opportunity for aquatic and marine ectotherms to behavioural thermoregulate, thus creating divergent selection based on the stability of water temperature. Therefore, thermoforming fish species have physiologically adapted to a wide range of temperatures, which is exemplified by 37.5% of studies mentioned in fish demonstrating no significant oxidative stress response to temperature changes, potentially leading to the conclusion that temperatures may not have been extreme enough. However, oxidative stress responses will be particularly strong when placing homeothermic organisms outside their preferred thermal range (Pamplona and Barja 2011). For example, deep-sea Antarctic fish experience minimal changes in oceanic temperature throughout their lifetime or, more importantly, their evolutionary history. When such species are exposed to acute temperature treatments as little as 4 degrees from optimal temperature, a strong oxidative response is observed (Almroth *et al.* 2015; Klein *et al.* 2017), revealing a narrow thermal performance breadth (*Figure 1*). In comparison, when thermoconformers, such as fish from estuarine and marsh environments (*Cyprinodon variegatus* and *Oryzias latipes),* are subjected to large temperature fluctuations in natal environments, they demonstrate minimal oxidative stress responses due to wider thermal breadth (Hemmer-Brepson *et al.* 2014; Baker *et al.* 2020). They exhibit very broad operative performance breadths (*Figure 1*). Thus, oxidative stress responses to temperature treatments are heavily dependent on phylogenetic and environmental contexts.

Acute versus acclimation studies

Acute and acclimation thermal treatments differ significantly in the physiological pathways they trigger, and therefore conclusions from these experiments are different. Acute thermal treatments demonstrate an organism's physiological ability to handle large fluctuations in temperature, whilst acclimation treatments test an organism's adaptive plastic capacity to respond to long-term thermal regimes. Acclimation treatments are more biologically relevant to long-term climate change questions, such as gradual changes in temperature predicted globally (IPCC, 2014). However, in some environments, sharp changes in thermal gradient, heat waves or cold snaps are expected to become more intense and frequent due to climate change (McKechnie and Wolf 2010). In which case, acute thermal treatments provide critical insight into the way species can handle such events. The study question must guide the type of thermal treatment used to test an organisms oxidative stress response to temperature for the results to retain biological significance. In conclusion, the phylogeny or more accurately, the thermal evolutionary history of a species or population, plays a crucial role in guiding oxidative stress responses in acute and acclimation experiments.

Candidate mechanisms to explain acute and acclimation responses

In acclimation treatments, 80% of treatments at cooler conditions demonstrated significantly greater oxidative stress than individuals acclimated at warmer temperatures (Ballen *et al.* 2012; Bury *et al.* 2018; Fitzpatrick *et al.* 2019; Dupoue *et al.* 2020)Interestingly, only one study (Bury *et al.* 2018) quantified responses in metabolic rates associated with the temperature treatment and thus provide strong evidence for oxygen consumption independent responses to oxidative stress. Traditionally, oxygen consumption has been thought to be one of the largest factors attributing to ROS production and the subsequent biological aging it produces. Large bodies of work have already elucidated these claims empirically within endotherms (Frisard and Ravussin 2006; Roberts and Sindhu 2009). However, some research has shown that, as oxygen consumption increases via increased metabolism, oxidative stress can decrease (Speakman *et al.* 2004; Salin *et al.* 2015a; Salin *et al.* 2015b; Bury *et al.* 2018; Koch *et al.* 2021). These results may explain the results of studies in which oxidative stress is lowered in warm acclimation treatments—providing a potential mechanism for ectotherms to mitigate warming scenarios successfully. However, the physiological mechanisms underpinning such phenomena are currently uncertain.

Temperature, through increasing metabolic rate, may lower ROS production via; mitochondrial uncoupling, elevated activity of antioxidant enzymes and upregulation of repair mechanisms. Mitochondrial uncoupling is any process in which electron transport is not used in ATP production and bypasses the use of oxygen substrate, which lowers membrane potential (Mookerjee, Divakaruni et al. 2010). When membrane potential is high, electrons are more likely to leak from the ETC, which is the primary way superoxide is formed (Echtay *et al.* 2002). As mitochondrial uncoupling generally occurs at elevated metabolic rates, it provides a viable explanation for alleviated oxidative stress at higher temperatures in ectotherms (Speakman *et al.* 2004). Conversely, at lower temperatures, mitochondrial dysfunction has been shown to increase (Chung and Schulte 2020) and the heightened production of ROS occurs (Christen *et al.* 2018).

Elevated activity of antioxidant enzymes and repair mechanisms may be another explanation for this phenomenon. In ectotherms, an increase in temperature can improve DNA repair efficiency (MacFadyen *et al.* 2004) and increase protein turnover (Houlihan 1991). The potential for certain antioxidant enzymes within ectotherms to have higher affinity at increased temperatures is possible, albeit under-explored (Sairam *et al.* 2000). However, due to the high specificity of enzymatic and non-enzymatic antioxidants, assessing their relation to whole organismal antioxidant capacity is challenging (Bury *et al.* 2018). When temperatures are below optimum, antioxidants may become less effective at quenching ROS, and transient times may become longer, thus increasing oxidative stress a low temperatures (Abele and Puntarulo 2004).

Finally, via the upregulation of repair mechanism through exposure earlier in the lifetime, organisms may alter the relationship between oxidative stress and temperature. Previous exposure can induce a hormetic effect, defined as the non-linear relationship between stressors and fitness (Calabrese 2013); meaning that increased mild exposures, through the acclimation process or in development, may be able to “prepare” the individual to functionally decrease oxidative stress later in life (Costantini, Metcalfe et al. 2010). Through the hormetic framework, we can hypothesise that environmental temperatures experienced in development may upregulate antioxidant defences and repair mechanisms to handle oxidative stress more effectively (Stahlschmidt, French et al. 2017). As a theoretical understanding of these mechanisms is in its blossoming stage, uncovering their biological implications in response to climate change may help predict the physiological effects on ectotherms.

Biological implications and recommendations

Understanding the adaptive potential of these mechanisms to buffer ectotherms from climate change effects may be crucial in understanding potential range shifts. The operating range and limit of their ameliorating effects, alongside the potential trade-offs they may incur, has the potential to alter species abundance and distribution through changes in life-history traits (Costantini 2008). As these proposed processes require additional energy to optimise fitness in changing environments, there is potential for trade-offs to incur, resulting in detrimental effects on cellular and organismal fitness. Additionally, the biogeographic and phylogenetic spread of these traits will also be of interest for understanding the impacts of warming across the range of a species.

To understand these effects, I propose some experimental procedures based on key studies mentioned in this review, which aim to uncover the role of temperature-effects on oxidative stress in ectotherms (Machado *et al.* 2014; Klein *et al.* 2017; Bury *et al.* 2018; Fitzpatrick *et al.* 2019). Acclimation treatments are of utmost importance to assess ectotherms' ability to deal with predicted climate change. Mimicking the effect of realistic sub lethal temperatures will produce biologically relevant results. These temperatures also need to be relevant to the optimal/preferred temperature of the study species. In stenothermal ectotherms, which are typically evolved to deal with thermally consistent environments (Pörtner *et al.* 2000), this means temperatures that are relatively close to the thermal optima. In eurythermal ectotherms, typically evolved in variable thermal conditions (Somero and Dahlhoff 2008), the experimental temperature changes may be much larger. Additionally, metabolic function is a crucial metric to measure as it provides a direct insight into the changes in oxygen consumption that acclimation experiments are predicted to induce. Traditional theory suggests that increased oxygen consumption is the primary source of ROS (Speakman *et al.* 2015), thus controlling for such factors will provide a significantly stronger argument against such ideas.

A pivotal study (Bury *et al.* 2018) provides a useful example of effective acclimation treatments with a biologically relevant selection of temperatures and metabolic function measurements. Grass snakes *(Natix natix)* were acclimated for six months to cool 18 °C and warm 32°C conditions; these temperatures were chosen as they fell outside of the species preferred range 24–27°C but lay just within their activity range (17–35°C). Therefore, individuals were acclimated to non-stressful conditions. Subsequently, standard metabolic rate (SMR) was measured alongside dROMS (reactive oxygen metabolites), OXY absorbent test (total non-enzymatic antioxidant capacity), providing a relatively complete view of the REDOX system. The cold-acclimated treatment was found to have significantly higher dROMS than the warm treatment despite lower SMR levels and comparable OXY levels. This experimental protocol provides a solid methodological framework for future research to be conducted.

Assessing the biogeographic variation in oxidative stress and telomere responses may help understand warmings physiological effects and the potential for subsequent changes in population abundance and distribution (Costantini 2008; Dupoué *et al.* 2017). whilst also uncovering the interrelatedness of oxidative and thermal stress responses. To achieve this, I recommend the survey of replicated wild populations, thermally adapted to different environments across a thermal cline in a reciprocal common garden experiment. If populations react similarly to temperature treatments, then thermal plasticity is the dominant factor. However, if populations respond differently to the treatments levels, thermal adaptation within the populations can be assumed to be high. A good example of evaluating biogeographic variation in oxidative stress and telomere responses can be seen in Fitzpatrick *et al.* (2019). The spotted snow skink *Niveoscincus ocellatus*, was sampled from four populations, two highland and two lowland, to assess the within and between oxidative stress differences in climatic regions. Populations were then spread evenly between a hot basking treatment (basking light for ten hours) and a cold basking treatment (basking light for four hours) for three months. Subsequently, cytoplasmic ROS and telomere length was measured. Within the cold-adapted lizards, ROS increased more in the cold treatment compared to the hot treatment, whilst in the warm-adapted lizards, no change was detected between treatments, additionally telomere length increased in the warm treatment. This study demonstrates that thermal adaptations may alter oxidative stress responses. Additionally, if populations are captured, bred and raised in captivity, then testing for environmental upregulation of repair mechanisms though the hormetic framework is possible. Exposure to increased temperature throughout different stages of development may demonstrate significantly less oxidative stress than control treatments kept at consistent ideal temperatures (Simčič *et al.* 2015). Alongside acclimation studies, the assessment of thermoregulatory behaviour throughout acclimation experiments is especially pertinent for terrestrial ectotherms. Changes in basking time indicate the behavioural modulation of internal body temperature (Carrascal *et al.* 1992). However, time spent basking in many ectotherms may also be linked to several other behavioural traits such as territory defence, mating and more generally bold/shy behaviour and therefore must be accounted for (Ballen *et al.* 2012; McEvoy *et al.* 2015; Horváth *et al.* 2017)

An appropriate selection of biomarkers will allow a sufficient understanding of the changes that occur to the REDOX system during acclimation. Several antioxidants' measures at multiple points throughout the acclimation process are advised to test for the temperature-dependent upregulation or activity of antioxidants. Additionally, if possible, multiple tissue samples will provide considerable detail on the distribution of oxidative damage around the body, thus highlighting potential impacts on fitness. Studies such as (Machado *et al.* 2014; Klein *et al.* 2017), provide excellent examples of this utilizing a wide variety of oxidative stress biomarkers. By measuring three oxidative damage metrics, and four metrics of antioxidant capacity, deeper analysis into correlation, within and between biomarkers was achieved. Studies such as (Klein *et al.* 2017) demonstrate the utilisation of multiple tissue samples. Brain, gills, liver, white muscle and erythrocytes were sampled with three oxidative damage markers and five antioxidants being quantified, providing information into the effects of temperature in oxidative stress on certain organ systems and, thus, which aspects of the physiology may be impacted.

Finally, to fully understand the role of temperature in oxidative stress in ecologically relevant settings, studies on wild populations and analysis of changes to life-history are crucial. Oxidative stress can influence life-history traits with flow-on effects to population and ecosystem structure (Monaghan *et al.* 2009). A distinct lack of studies on populations in their natural habits impedes the development of solid guidelines for ectothermic conservation. This fact is particularly important for invertebrates as there is a distinct lack of data around the role of oxidative stress in life-history traits and the potential for temperature to alter this relationship (Chainy *et al.* 2016). Thus, studies quantifying life-history changes across a wide range of populations and phylogenetic groups are predicted to provide the most useful information in understanding the potential physiological costs of warming.

Conclusion

Temperature increases associated with climate change have the possibility of altering oxidative stress processes, life-history traits and population structure in ectotherms. However, there is a significant need for increased ecological, biogeographical, and evolutionary studies on the role of temperature on oxidative stress and subsequent changes in life-history traits in wild populations. The investigation of oxidative stress responses at physiological and behavioural levels is crucial in providing valuable predictions. A deeper understanding of the effects of temperature on oxidative stress in ectotherms may increase knowledge to predict climate changes' impact on ectotherms and biodiversity.

Thesis Structure

The two data chapters of this thesis (Chapters 2 & 3) are presented as separate scientific papers prepared for potential publication. As such, chapters may contain some unavoidable repetition in terms methodological descriptions of field collection and animal husbandry.

Chapter 2:

Adaptation or plasticity? Effects of temperature on metabolic rate and life-history traits in the Australian Painted Dragon lizard.

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Abstract

The role of adaptation and plasticity in shaping life-history traits has been a long-standing debate in evolutionary ecology, with the relevance of each being highly specific to traits, populations, species and clades. Ectothermic organisms, who gain the majority of their metabolic energy from ambient heat, occupy wide geographic ranges in which heat can be unevenly spread. There is considerable potential for populations at opposite ends of a temperature-cline to exhibit differing thermal adaptations, energy budgets and phenotypically plastic responses to temperature. Using factorial laboratory experiments, we investigated the hypothesis that two populations of male painted dragon lizards (*Ctenophous pictus*) would significantly differ in thermal physiological traits from opposite ends of a temperature-cline. After acclimatising both populations in a warm and cool temperature treatment, we quantified differences in; thermal performance curves, somatic growth (body size and weight) and reproductive investment (relative testes mass). We found that the Northern, warm adapted population had a significantly higher metabolic rate at high temperatures and had greater investment in body size and relative testes mass than the cool adapted southern population, consistent with geographic variation of thermal traits in both populations. The effects of temperature treatment were negligible except in growth in body length (skeletal growth), which was greater in the cool treatment for both populations, hinting at similar thermal plasticity response levels. Our results suggest that thermal adaptation and phenotypic plasticity both play a role in determining life-history variation in *C. pictus*.

2.1. Introduction

Geographic variation is a valuable way to study the fundamental processes of adaptive evolution (Pörtner 2002b). Life-history traits are essential components of fitness (Ricklefs and Wikelski 2002). Changes in phenotypes along environmental clines form via variable trade-offs due to finite resources and the effect of those resources on metabolic processes (Ricklefs and Wikelski 2002). Temperature has a profound impact on all organisms' metabolism but especially in ectotherms, whose metabolic rate is primarily driven by environmental sources of heat (Angilletta 2009). Thus, ectothermic species spread over a broad geographic range evolve contrasting thermal and metabolic adaptations to adjust to local conditions (Angilletta 2009). When local environmental conditions are consistent, the optimisation of metabolic and cellular processes can be incredibly fine-tuned (Flukes *et al.* 2015). However, when conditions are highly variable, metabolic and cellular processes must be plastic, allowing individuals to live in various conditions (Schulte *et al.* 2011). Both these evolutionary pathways can play a role in determining the speed and efficiency of metabolic functions, which can ultimately lead to variation in life-history traits as a function of temperature across a species' range (Angilletta *et al.* 2004).

Ectotherms are metabolically sensitive to changes in ambient heat levels and have developed a host of thermal adaptations to optimise bodily function in various environments via behavioural and physiological means (Angilletta 2009). One way of quantifying differences in thermal adaptations is via measuring the resting metabolic rate (RMR) of an organism (Marshall and McQuaid 2011). Resting MR in ectotherms is defined as an organism's metabolic rate in a quiescent, post-absorptive state; it is the basic energetic cost of self-maintenance at a given temperature (Burton *et al.* 2011). High RMR within individuals suggests increased capacity for energetically costly activities (metabolic scope) such as sprint speed, territorial defence, somatic and reproductive investment and are often linked with fitness (Cano and Nicieza 2006). In ectotherms, thermal metabolic optimum should evolve to match the most prevalent temperatures a population experiences when resources are most available (Seebacher 2005). However, high RMR is energetically costly, and the fitness benefits of maintaining high RMR depend on resource availability (Huey and Kingsolver 2019). Therefore, when conditions are highly variable, plasticity in metabolic rate is advantageous (Seebacher and Franklin 2011). However, not all species, populations or individuals are equally plastic. For example, cane toads from higher latitudes living in higher seasonal and diel environmental variability exhibit greater plasticity in metabolic rate than individuals from lower latitudes where temperatures are more uniform (Winwood-Smith *et al.* 2015). Thus, selection will favour high and consistent RMR when conditions are stable, and plastic RMR when conditions are unpredictable (Seebacher and Franklin 2011). Changes in optimal metabolic temperatures and plasticity due to environmental variability can alter selection between life-history traits such as somatic investment and reproduction (McGill *et al.* 2006).

Metabolism associated with somatic investment and growth form a significant portion of an organism's energetic budget and therefore are reliable indicators of inter-population differences in resource availability and allocation (Adolph and Porter 1993). In ectotherms, a substantial proportion of growth rate and growth period is believed to be a heritable trait and an evolved adjustment to environmental conditions (Chandan *et al.* 2019). Fast and consistent somatic growth directly correlates to body size, which scales to nearly all facets of the phenotype such as morphological, behavioural, reproductive, physiological and life history characteristics (Honěk 1993; Johnsson *et al.* 1999). Large body size is achieved via increases in skeletal, muscular, organ weight, and fat stores throughout an organism's lifetime and significantly increases longevity (Adolph and Porter 1993). However, somatic growth rate in ectotherms is highly plastic, alterations in temperature and food consumption (i.e. changes in metabolic scope) in early-life have strong influences upon growth efficiency (Sibly and Atkinson 1994). Additionally, many ectotherms such as fish, amphibians and reptiles have indeterminate growth, continuing plastic growth rates throughout the entirety of an organism's life (Dumas and France 2008). Due to the cost of growth, some reptiles are known to decrease in mass and skeletal length when conditions are unfavourable and increase when conditions improve (Wikelski and Thom 2000; Chiari *et al.* 2016). Growth and somatic investment can reflect adjustments to resource availability over evolutionary time-scales and within individuals lives (Adolph and Porter 1993). Alterations in metabolic function caused by disparate resources may also influence life-history traits such as reproductive investment in metabolic function (Pörtner 2002b).

Investment in current somatic growth versus investment in future reproduction is the most basic life-history trade-offs in nature and varies between populations when resources are disparate (Pörtner 2002b). Investment in future reproduction increases the chances of successfully passing on genes; however, if investment in reproduction is too energetically costly, somatic growth and repair mechanisms will be impaired (Cox *et al.* 2010). This trade-off is sensitive to environmental temperature changes that can tip the balance by affecting energetic budgets (e.g., food availability and metabolic rates), shifting resource allocation strategies along environmental clines (Urbanski *et al.* 2012). In populations subject to an unpredictable environment, selection will often favour early sexual maturity and quick reproduction (i.e., bet-hedging (Mitchell 1988), or opportunistic strategies (Roff 1993)), which requires high levels of metabolic expenditure early in life. Whilst in stable environments, selection favours significant somatic growth, increased size at maturity and a relatively lower metabolic rate with energetic expenditure on somatic repair mechanisms (Roff 1993). In addition to the effects of environmental stability on somatic investment, large body size is also regularly favoured by sexual selection (Wikelski 2005). Large individuals are often more fecund and better able to defend territories (Wikelski 2005). As body size is energetically costly, it cannot be faked and acts as an honest sexual signal which becomes a primary trait used by females to assess male mate quality (Wikelski 2005). Similarly, investment in sexual colouration is a costly reproductive investment, used to demonstrate mate quality in various species (Grafen 1990; Servedio and Boughman 2017). Therefore, alterations in metabolic activity due to disparate resources between populations can change levels of reproductive and somatic investment via tradeoffs. Therefore, we assess the relative effects of adaptation and plasticity on: body size, reproductive investment, growth and metabolism in two populations of the Australian painted dragon lizard (*Ctenophorus pictus)* from opposite ends of a temperature-aridity cline acclimated to warm and cool conditions.

Ctenophorus pictus is a well-studied colour-polymorphic lizard native to a large portion of southern central Australia. Most detailed investigations of *C. pictus* have been conducted at a single population in Yathong reserve, NSW; thus, background knowledge of this species is primarily population-specific. In the Yathong population, RMR is morph-specific; individuals possessing a yellow gular patch or 'Bib" have a significantly higher metabolic rate than those without (Friesen *et al.* 2017a; McDiarmid *et al.* 2017). Higher metabolic rates have been linked to sexual selection, with bibbed males being more aggressive (McDiarmid *et al.* 2017), defending more territory (Healey and Olsson 2009), thus requiring increased metabolic scope. As *C. pictus* is an annual lizard (roughly 80-90% die before reaching one year of age), the species, at least in the Yathong population, takes an opportunistic strategy with a short lifespan and early maturity indicative of evolution in an unpredictable environment (Olsson *et al.* 2007b). However, the two populations used in this study occupy vastly different abiotic environments in terms of thermal variability, and seasonality thus providing an opportunity to investigate the effects of geographic variation in thermal adaptation and life-history traits. To assess the relative effects of adaptation and plasticity in thermal physiological traits, we housed adult males from both populations in warm and cool treatments for 3-months. Subsequently, body size, growth, reproductive investment and metabolic rate were used as indicators of adaptation (population effects), plasticity (temperature treatment effects) or the interaction of both within this species. We predict that the warm/variable adapted population will be larger in body size, grow faster and invest more in reproduction due to increased energetics associated with warm conditions, these differences will further be enhanced in the warm treatment (Cano and Nicieza 2006; Angilletta 2009). Furthermore, we predict that the warm and variable adapted population will have a higher and more plastic metabolic rate and critical maximum temperature than the cooler and stable adapted population due to the higher temperature variability experienced evolutionarily.

2.2. Methods

2.2.1 Animal collection/housing

The Australian painted dragon lizard (*Ctenophorus pictus*) is a small, diurnal, Agamid lizard (Healey and Olsson 2009) with a broad distribution across south-central Australia (Cogger 2014) (*Figure 3*). Lizards were caught in October 2019 from two discrete field sites in South Australia with hypothetically zero gene flow between them due to habitat fragmentation: Muloorina station (29°14'; 137°54') to the north, and Innes National Park at the tip of the Yorke Penninsula (35°14'; 136°54') to the south (*Figure 3*). These field sites were chosen as model arid and coastal populations because of large differences in average annual rainfall with average yearly rainfall approximately three times lower at the northern site (350 mm vs 125 mm annually). Additionally, the Southern site is on average 8-10°C colder, and the average number of days over 40°C can be ~15 times less in the south during the breeding season of October to December (*Figure 2*). Furthermore, average daily temperature variation (high-low) in the north is double that of the south, and inter-season variability is considerably less in the south (*Figure 4*). As such, the northern site is classed as markedly hotter and more variable than the southern site.

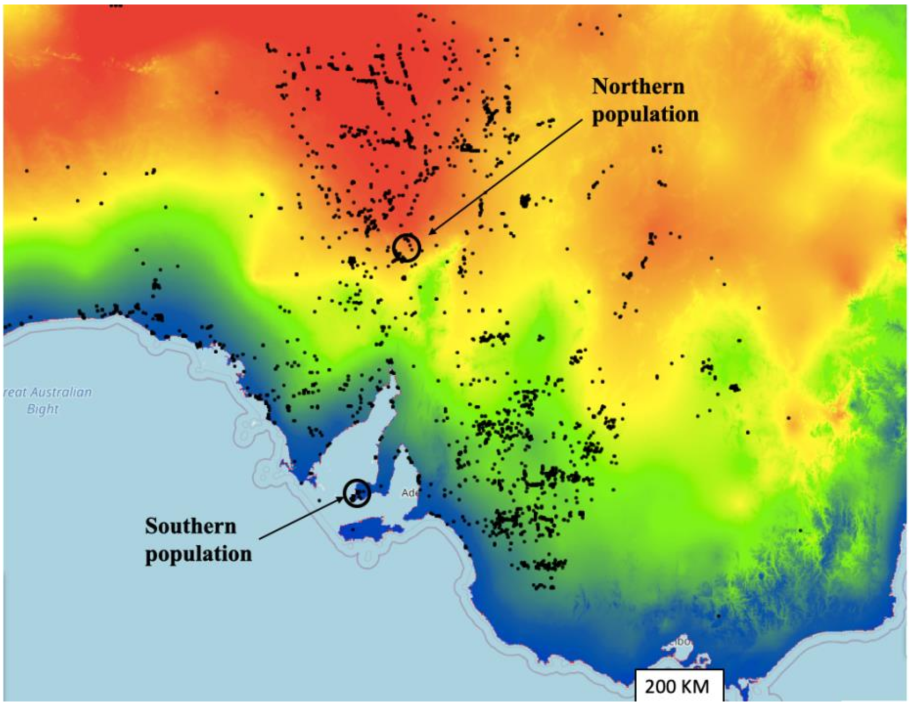

Figure 3. *Map of field sites in which populations of* C.pictus *were sourced; Muloorina station (Northern population) (29°14'; 137°54'), and Innes National Park (Southern population) (35°14'; 136°54'. Black dots indicate records of* C. pictus *from Atlas of Living Australia (https://www.ala.org.au/). Colour shading in the map indicates temperature/aridity (orange: high aridity, and blue: low aridity). Black dots indicate records of* C. pictus *sightings*

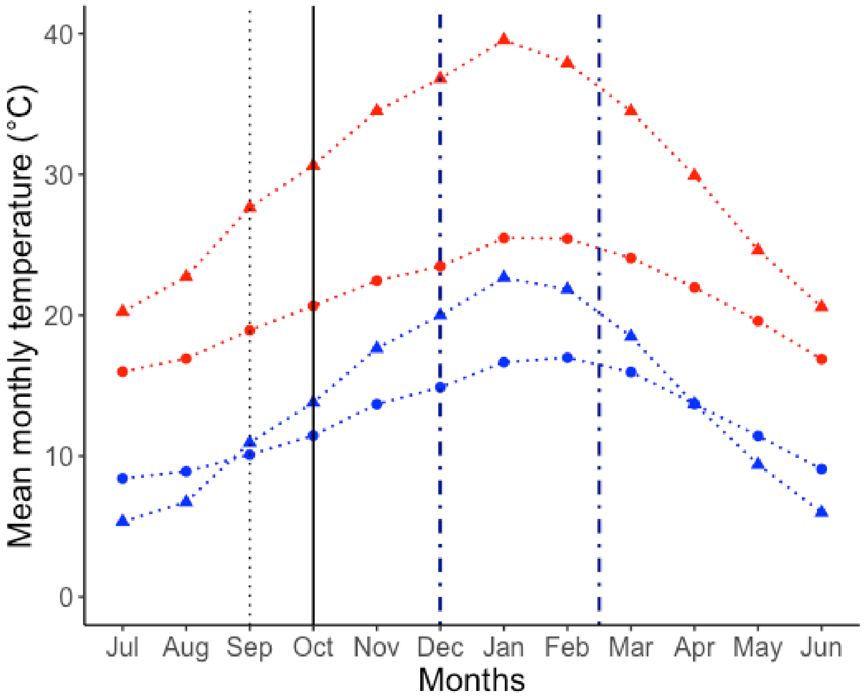

Figure 4. *Average daily High (red dotted line) and Low (blue dotted-line) temperatures for each month over the years 1996-2015 for the two locations; Northern population (Triangles) data from Marree, SA weather station. Southern population (Circles) data from Stenhouse Bay, SA weather station. Vertical lines indicate the presumed beginning of breeding season for the Northern population (dotted line) and Southern population (solid line). Climate data sourced from the Bureau of Meteorology. Vertical dot-dash lines indicate study period.*

All animals were sexed, weighed, inspected for bib presence/absence and assigned an ID upon capture via hand and lasso (total N = 40 males, North N=21, South N=19). Females were excluded due to uncertainty about the staging of their asynchronous reproductive cycles both within and between populations (Ibargüengoytía and Boretto 2006). Additionally, we were interested in the associations of bib (sexual selection) on response variables. Lizards were transported to the University of Wollongong. The lizards were housed individually in opaque 88L enclosures (400 x 400 x 620 mm) with a heat lamp at one end to allow thermoregulation. Males from each population were randomly spread across two separate rooms (cool, N = 20; warm, N = 20). Ambient baseline and over-night temperatures in both rooms were set to 15C. To form the temperature treatments, heat lamp wattage was manipulated: One room had 40 W heat lamps whilst the other had 60W heat lamps, which generated different ambient temperatures in each room (warm mean = 24.743, ±SE = 0.093, cool mean = 20.366, ±SE = 0.055) and thus termed warm and cool treatments hereafter. Max mean temperatures under the heat lamps within the enclosures matched average maximum temperatures at both the Northern and Southern populations (Max mean tub temperature warm room = 42.5 °C, ±SE = 2.2; Max mean tub temperature cool room = 28.5 °C, ±SE =1.5, *Figure 1*). The lizards were kept in these temperature treatments for a 3-months. Each room's UV and ambient lights were set to a 10 h:14 h Light: Dark cycle, and heat lamps were set to a 7 h:17 h on: off regime. The animals were fed mealworms dusted with calcium and pre-formulated multivitamin powder and misted with water every second day.

2.2.2 Experimental procedures

2.2.2.1 Metabolism

Metabolism measurements were taken for three sets of 12 animals and one set of four animals. Each set of animals were fasted for 72-hours before measurements. The night before RMR measurements, the animals were collected from their home enclosures after the heat lamps had been turned off and ambient temperatures in the room had dropped below 18 °C. Animals were weighed, measured and placed in respirometry chambers of known volume (Mean chamber volume = 810ml, ±SE = 4 ml). The chambers were then placed in an incubator and stepwise cooled through an acclimation period of half an hour in 5 °C increments until they reached the planned starting temperature of 10 °C, at which they equilibrated for 60 minutes with valves of the metabolic chamber open. After equilibrating, the chambers were flushed with fresh air and returned to the incubator overnight (with the valve now closed to capture respired air). At the end of the measurement period, one 50 ml air sample was taken from each chamber through valves with calibrated syringe, which was immediately sealed with an air-tight cap until injecting the sample into the inlet tube of Sable Systems FMS oxygen analyser (Sable Systems, International, North Las Vegas, NV), with flow-rate set to 250ml/min to measure VO^2 (oxygen consumption as ml min^{-1}). Oxygen concentration output was recorded by Warthog LabHelperX software (build 23, October 2015, 1989–2016 Mark A. Chappell and the Regents of the University of California). Lizard mass was used as a proxy for the lizard's volume in calculations of total air volume in the chamber, which is highly correlated with volume estimated based on the water-displacement method, and is more accurate and precise than water-displacement ($R^2 = 0.957$, n = 35, thus 1g ≈ 1ml) (Friesen *et al.* 2017a). After the air samples were collected from all the animals at a given temperature, the incubator temperature was raised 5 °C to the next measurement temperature. The chamber was flushed to restore O^2 levels, and the valve was left open for 30 minutes before it was closed again to start the next measurement period. This procedure was repeated every 5 °C (10-40 °C). Each measurement period was timed and recorded ± 1 s. Measurement periods were tailored to each temperature to ensure O^2 was detectable by the oxygen analyser on pilot data whilst also limiting the time lizards time spent at high temperatures (10 °C = 875 min; 15°C = 165; 20°C = 105 min; 25°C = 85 min; 30°C =70 min; 35°C = 50 min; 40°C = 40 min).

Oxygen consumption was determined by integrating the area under the curves from the output graphs, O^2 (Lighton 2018) in Warthog LabAnalystX (build 15, October 2015, 1989–2016 Mark Chappell and the Regents of the University of California). Total O^2 consumption of the lizard was calculated as (Friesen *et al.* 2015; Lighton 2018), $V_{lizard} = V_{measured} * (V_{chamber} / V_{sample})$, where V_{lizard} is the total volume ml O^2 consumed by the lizard during the time in the chamber; $V_{measured}$ is the volume (ml) O^2 change in the sample calculated by integrating the peak; $V_{chamber}$ is the chamber volume minus the lizard volume (1g ≈ 1ml), and V_{sample} is the sample volume (50 ml). The 0^2-ml min^{-1} were calculated as $\dot{V}O^2 = V_{lizard} / t$, where t is time in the enclosure in seconds (± 1s).

2.2.2.2 Basking behaviour

Basking score was tabulated roughly 2.5 times a week over the three-month acclimation period by entering the treatment rooms at various times throughout the day and noting each individual's location within their tub in a binomial fashion (e.g. 1 = basking, 0 =

not basking). The time at which measurements were taken were divided into six periods of the day for ease of analysis. the categories are as such: Before heat lamps (8:30am-9:00am); = Early morning (9:00am-10:30am); Late morning (10:30am-12:00pm); = Noon (12:00pm-1:30pm); Afternoon (1:30pm-2:30pm); Late Day (2:30pm-4:30pm).

2.2.2.3 Critical temperature maximum.

In ectotherms, thermal optimum (Topt) is the ideal temperature for physiological functions, whilst critical temperatures, both high (CTmax) and low (CTmin), are the functional limits of metabolic scope (Pörtner 2002). Measuring critical maximum temperature (CTmax) as the temperature when mouth-gaping occurs avoids potential harm associated with "traditional" methods, usually determined by the point at which loss of righting response occurs (Taylor *et al.* 2020). Lizards were fitted with a small thermocouple ~3 mm in their cloaca, held in place by adhesive tape (3M Nexcare^{TM}), allowing the exact body temperature when CTmax is reached to be recorded. Lizards were heated until mouth gaping occurs, heating stopped, temperature recorded, and lizards were observed as they returned to their preferred body temperature and placed back in their home enclosure.

2.2.2.4 Dissection and tissue collection

After the 3-months, the lizards were euthanised to assess testes size alongside tissue samples required for another experiment. The lizards were heavily sedated via an injection of Zoletil (tiletamine/zolazepam); once sufficiently sedated as determined by loss of righting-response, they were euthanised with an overdose of sodium pentobarbitone and decapitated to ensure death prior to dissections. Subsequently, body size in mass (±0.001 g) and SVL (±0.5 mm) were measured alongside, testes weight (±0.001 g) were recorded. Testes size was used as a robust proxy for male reproductive investment as postcopulatory selection regularly favours large testis size relative to body mass (Todd 2008; Uller *et al.* 2010; Lüpold *et al.* 2020). Animals were collected under a scientific research license issued by South Australia Department of Water and Environment permit (A26776-1-4), and the collection and experiments were conducted in accordance with research authority granted by University of Wollongong Animal Ethics Committee (AE1907A04).

2.2.3 Statistical methods

2.2.3.1 Morphology

All statistical analysis was conducted in R studio (RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Body condition index (BCI) was calculated as the standardised residuals (mean = 0; standard deviation = 1) from a linear regression of ln(body mass) as a function of ln(SVL) per population at first time in the lab ($\mathrm{BCI}_{\mathrm{initial}}$) and time of euthanasia ($\mathrm{BCI}_{\mathrm{final}}$) (Labocha *et al.* 2014). To model differences in $\mathrm{BCI}_{\mathrm{final},}$ mass (g), SVL (cm) and reproductive investment at time of euthanasia, separate ANOVAs were constructed with the explanatory variable of Population (Northern v Southern). Reproductive investment was evaluated by calculating the percentage of testes mass relative to body mass (testes mass/body mass x 100), termed the Gonadal somatic index (GSI) (Todd 2008; Uller *et al.* 2010; Lüpold *et al.* 2020). To assess population, temperature treatment and bib effects on the relationship between $\mathrm{BCI}_{\mathrm{final}}$ and GSI, separate ANCOVAs were run. Temperature treatment and bib showed no correlation, however population was significant. Subsequently, two linear regressions were used to determine each population's relationship between BCIe and GSI. Finally, the region of non-significance was determined using the Johnson-Neyman technique (White 2003).

2.2.3.2 Growth

Growth was calculated as the percentage change from the first measurements in the lab to time at euthanasia for both body mass (±0.001 g) and SVL (±0.5 mm). Separate ANOVA's were constructed with Population, Temperature treatment and their interaction as explanatory variables for both response variables of body mass and SVL growth. The relationship between $\mathrm{BCI}_{\mathrm{initial}}$ and the two response variables of growth, body mass and SVL, were modelled in separate linear regressions.

2.2.3.3 Basking behaviours

We constructed a Generalised Linear Mixed effect models (GLMM, using the R package "lme4" " (Bates *et al.* 2007) to test for significant differences in Basking score using the fixed effects: temperature treatment, Population and time of day plus the three-way interaction between these variables and Male ID as a random effect. The GLMM family used in the basking score analysis was binomial with a logit function and weighted to a value of 1 per observation. Posthoc pairwise comparisons were conducted with the package 'lsmeans" (Lenth and Lenth 2018) to attain and test which combinations of Population, temperature treatment and time of day were significantly different (Tukey adjusted p-values to account for multiple, posthoc comparisons).

2.2.3.4 Metabolism

Mass specific metabolic rate (hereafter called msRMR) was calculated as O^2 consumption rate (ml/min) divided by the lizard's mass at time of measurement (±0.001 g). Subsequently, msRMR was $\log_{10}$ transformed to improve normality and homogeneity of variance. A linear mixed model (LMM) using the R package "lme4" " (Bates *et al.* 2007) was constructed with Temperature (of msRMR measurement, treated as a discrete, categorical variable), Population and thermal treatment as explanatory variables, plus the interaction terms of Temperature* Population, Population* thermal treatment, Temperature* thermal treatment and ID as a random effect (to account for repeated measures at different temperatures). However, thermal treatment was not significant and replaced by Bib presence/absence, due to significant effects on msRMR in previous investigations in this species (Friesen *et al.* 2017a), mildly improve model fit ΔAICc = -3.05 (Konishi and Kitagawa 2008). Thus, the final LMM used Temperature, Population and Bib as explanatory variables plus the interaction terms of Temperature* Population, Population*Bib, Temperature*Bib and ID as a random. The results of the final msRMR LMM were assessed using a Type III, sums of squares (SS) ANOVA using Satterthwaite's method within the package "lmertest" (Kuznetsova *et al.* 2017). R^2 of final msRMR LMM was calculated using the R package "MuMIn" (Barton and Barton 2015) and posthoc pairwise comparisons were conducted using the R package "lsmeans" (Lenth and Lenth 2018) to assess differences between groups using Tukey adjusted p-values to account for multiple comparisons. CTmax was modelled as a function of Population and temperature treatment and their interaction in an ANOVA. The significance level for all tests was $\alpha = 0.05$. Graphs were constructed using the R package "ggplot2" (Wickham 2016). R packages "tidyverse" (Wickham 2017) and "dplyr" (Wickham et al. 2015) were used for data handling and manipulation.

2.3. Results

2.3.1 Morphology and reproductive investment

Significant differences between populations were found in Mass (g), SVL (mm) and GSI (%). The Northern population was larger in mean body mass, SVL and GSI (*Table 2*). However, BCI$_{final}$ did not differ between populations. Bib frequency differed between populations, with the Southern population being 52% bibbed and the northern population being 38% bibbed. Population significantly affected the relationship between BCI$_{final}$ and GSI ($F_{1,37} = 98.08$, $P < 0.001$), with the lower bound of the region of non-significance being 1.919, which is well above the largest values of BCI$_{final}$ (*Figure 5)*. Body CI$_{final}$ and GSI were positively correlated in the Southern population ($F_{1,17} = 9.937$, $P = 0.006$, $R^2 = 0.33$) whilst the Northern population had a non-significant negative relationship ($F_{1,19} = 3.724$, $P = 0.0687$, $R^2 = 0.12$, *Figure 5*).

Table 2*. Per population mean and standard error of lizard mass (g), SVL (cm), GSI (%) and BCI. Significance derived from individual ANOVA's for all four variables.*

		Body Mass (g)	SVL (mm)	GSI (%)	BCIf$_{inal}$
Northern Population	**Mean**	13.062	71.718	1.302	0.092
	Standard error	±0.391	±0.687	±0.066	±0.211
Southern population	**Mean**	9.942	64.631	0.396	-0.102
	Standard error	±0.479	±0.852	0.062	±0.241
	Significance	**≤0.005 ***	**≤0.005 ***	**≤0.005 ***	**0.548**

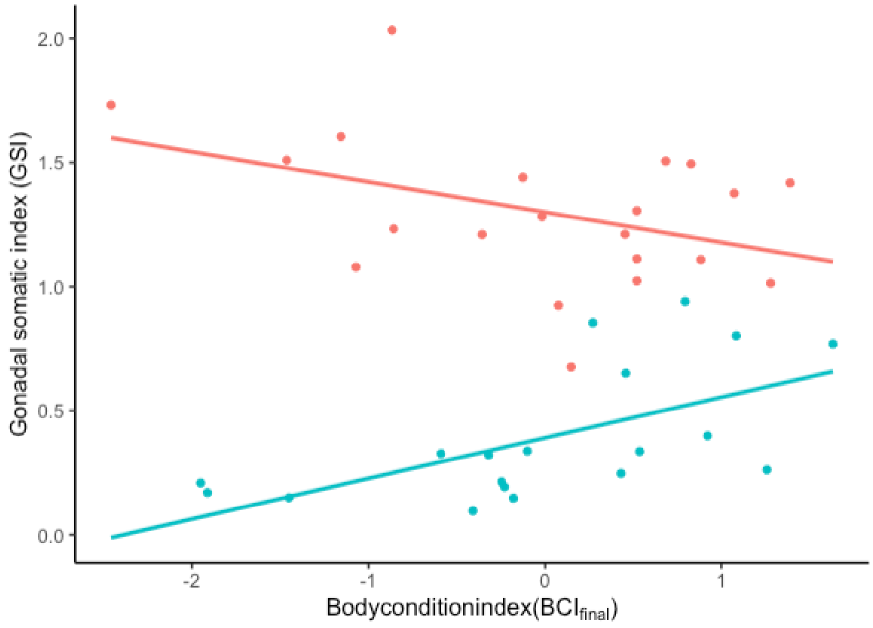

Figure 5*. Scatterplot showing contrasting correlations of population specific body condition (BCI$_{final}$) and gonadal somatic index (GSI) from two populations of* C. pictus, *North (Red) and South (Blue). Coloured lines depicting population regression lines (Southern population ($F_{1,17} = 9.937$, $P = 0.006$, $R^2 = 0.33$), Northern population ($F_{1,19} = 3.724$, $P = 0.0687$, $R^2 = 0.12$)), which significantly differed from each other across all realistic values of BCI$_{final}$ values >1.917.*

2.3.2 Growth

SVL growth as a percentage of initial SVL over the three-months differed between temperature treatments ($F_{1,35}$ = 7.701, P <0.001), but not Population ($F_{1,35}$ = 0.050, P = 0.824), Bib ($F_{1,35}$ = 0.283, P = 0.598), and did not depend on the interaction between Population and temperature treatment ($F_{1,35}$ = 0.146, P = 0.705) (*Figure 6A*). Individuals kept in the cool treatment had significantly greater SVL growth (%) (mean = 4.723, ±SE = 0.557) than those held in the warm treatment (mean = 2.544, ±SE = 0.511) regardless of population (*Figure 6A*). SVL growth (%) was strongly positively correlated to $BCI_{initial}$ ($F_{1, 38}$ = 20.393, P < 0.001, R^2 = 0.334). Mass growth as a percentage of initial mass over the three-months did not differ between Populations ($F_{1,35}$ = 0.572, P = 0.454), temperature treatments ($F_{1,35}$ = 0.436, P = 0.513), Bib ($F_{1,35}$ = 0.030, P = 0.846) and did not depend on the Population* temperature treatment interaction ($F_{1,35}$ = 0.038, P = 0.846) (*Figure 6B*). Non-significance may be attributed to small sample sizes relative to the large amount of individual variation (*Figure 6B*). There was no relationship between BCI_{inital} and mass growth ($F_{1, 38}$ = 1.874, P = 0 .179, R^2 = 0.022).

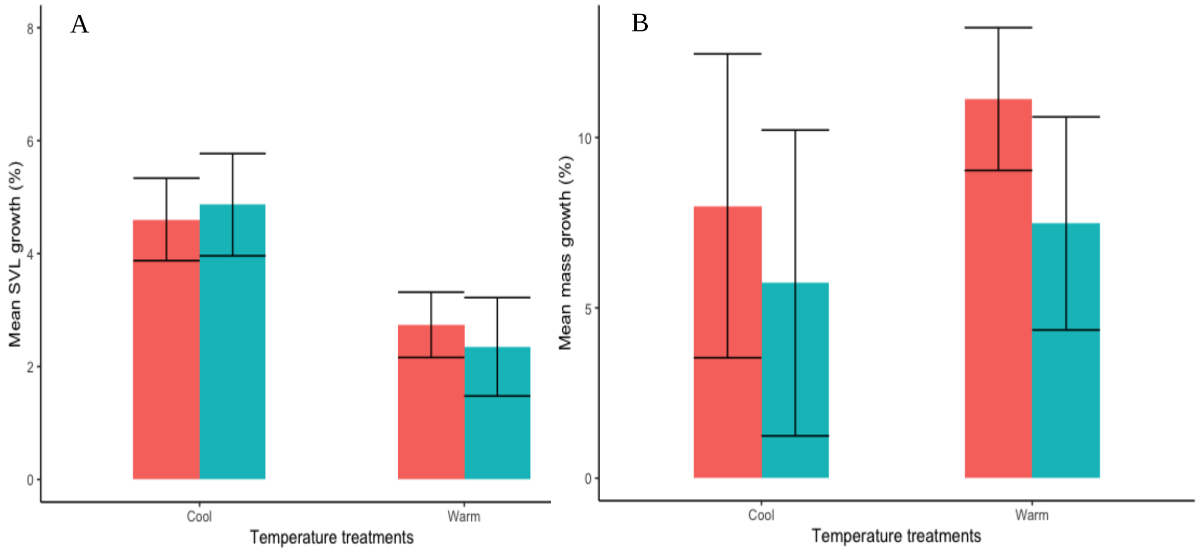

Figure 6**. Growth (%) of* C. pictus *males from Northern (Red) and Southern (Blue) populations that were kept in warm and cool treatments over a 3-month period. Error bars represent ± SE.* ***A. *mean SVL growth (%). Temperature treatment was found to be significantly different, whilst populations showed no significant differences.* ***B.*** *Mass growth (%). No significant differences between temperature treatment or population were found.*

2.3.3 Basking behaviours

In the warm treatment, significantly more males from the northern population (Mean proportion = 0.927, ±SE = 0.032) were basking only in the 'afternoon' than males from the southern population (Mean proportion = 0.4844, ±SE = 0.063) (*Figure 7*). In the cool treatment, the northern population basked considerably more (Mean proportion = 0.666, ±SE = 0.092) in the 'afternoon' than the southern population (Mean proportion = 0.069, ±SE = 0.048), and in the 'late day', with northern males (Mean proportion =0.532, ±SE = 0.042) basking more than southern males (Mean proportion = 0.310, ±SE = 0.039) (*Figure 8)*.

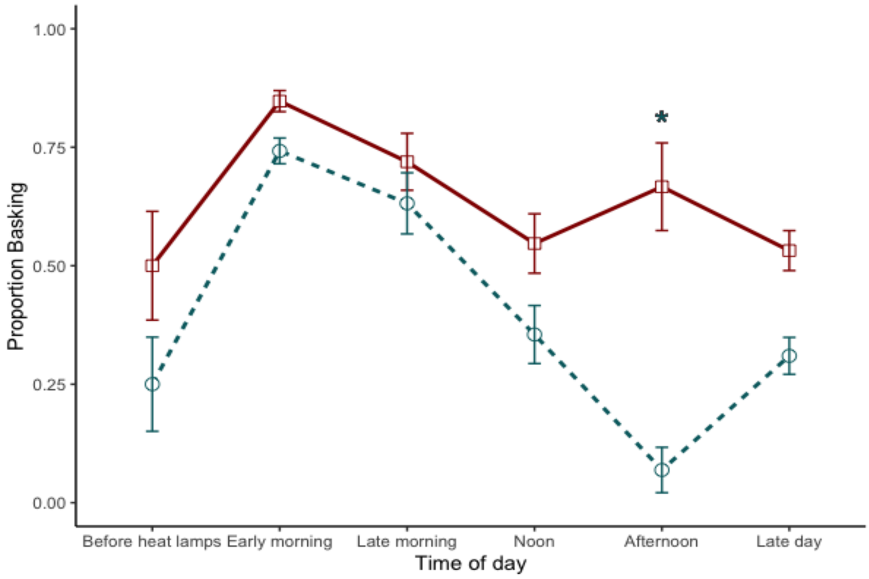

Figure 7. *The average proportion of* C. pictus *males basking throughout the day in the warm treatment. The Northern population (solid red lines), the southern population (blue dashed lines). Error bars depicting ±SE of the mean proportion in each population. Asterisk (*) depicting significance in a posthoc pairwise comparison*

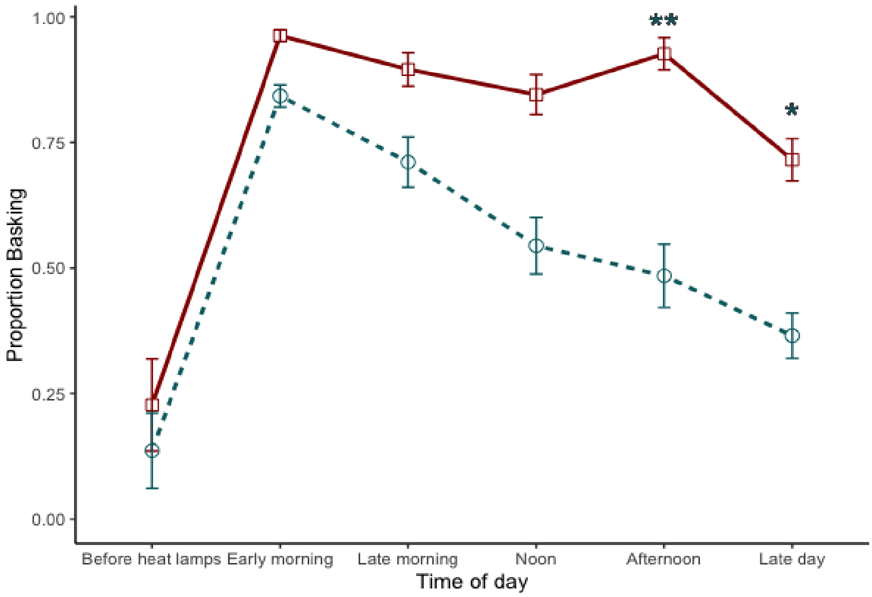

Figure 8. *Average proportion of* C.pictus *males basking throughout the day in the cool treatment. The Northern population (solid red line), the southern population (blue dashed line). Error bars depicting ±SE of the mean proportion in each population. Asterisk (*) depicting significance in a posthoc pairwise comparison*

2.3.4 Metabolism

Mass specific RMR significantly increased with temperature from 10-40 °C ($F_{6,222}$ = 916.808, P < 0.001). There was a significant Population* Temperature interaction ($F_{6,222}$ = 2.8072, P = 0.012), with the northern population having a higher RMR at most temperatures but only significantly at 40 °C (*Figure 9*). Bib was not significantly associated with msRMR ($F_{1,40}$ = 0.677, P = 0.415) nor were any interaction terms *(Table 3*). Individual rank repeatability was relatively high throughout temperatures (R^2 = 0.413, 95% CI = 0.275-0.569, P < 0.001). CTmax was statistically different between populations ($F_{1,32}$ = 26.420, P < 0.001) but not temperature treatments ($F_{1,32}$ = 0.677, P = 0.417) nor interaction term ($F_{1,32}$ = 0.658, P = 0.423), with the Northern population having a higher CTmax (mean = 41.0, 95% CI = 40.721-41.279) than the Southern population (mean = 38.9, 95% CI = 38.615-39.185, *Figure 10*).

Table 3. *msRMR. Results of type III SS ANOVA output of LMM using Satterthwaite's method. Bold text in the p-value column indicates significance at P ≤ 0.05.*

Full model R^2	0.954					
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**P-value**
Temperature	77.962	12.9937	6	222.171	916.808	**<0.001**
Population	0.025	0.0246	1	36.401	1.735	0.196
Bib	0.010	0.0096	1	40.588	0.677	0.415
Temperature*Population	0.239	0.0398	6	222.191	2.807	**0.012**
Population*Bib	0.030	0.0304	1	40.588	2.144	0.151
Temperature*Bib	0.163	0.0272	6	222.276	1.918	0.079

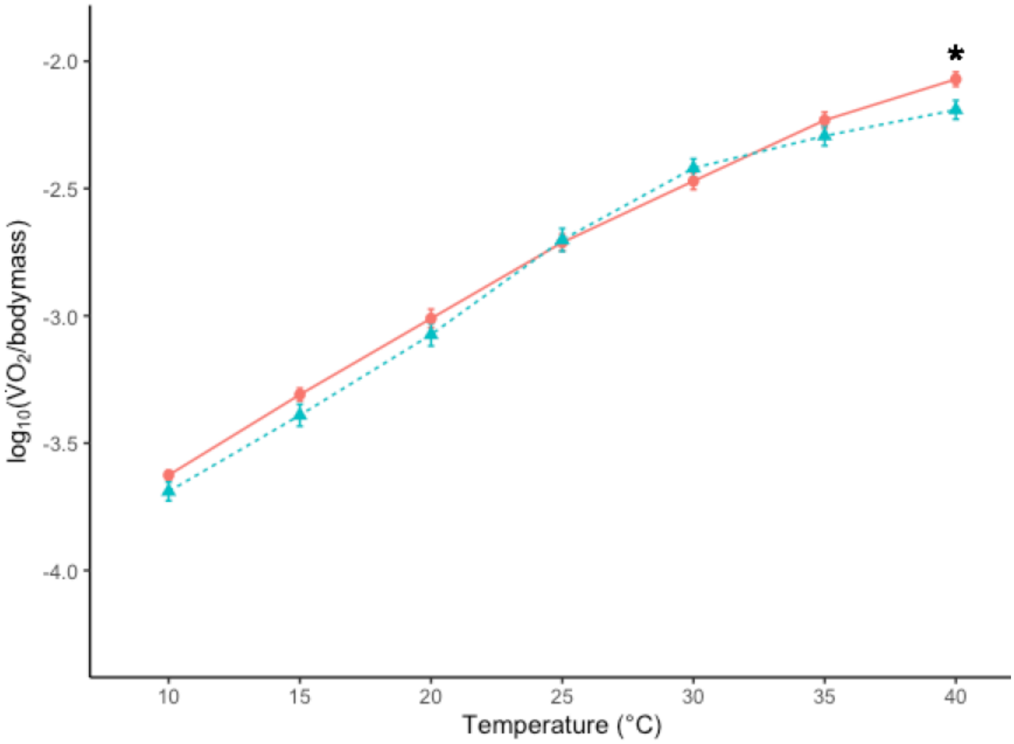

Figure 9. *Mean values of RMR (log*$_{10}$ *V̇O2/body mass) of adult male* C. pictus *from the Southern population (Blue triangles) and Northern population (red circles) at seven increasing temperatures (°C). The northern population has a significantly higher msRMR, particularly at 40 degrees. Error bars represent ±1 SE. Asterisk depicting significance in a posthoc pairwise comparison of an LME which used Temperature*Population as the interaction term *α ≤ 0.05.*

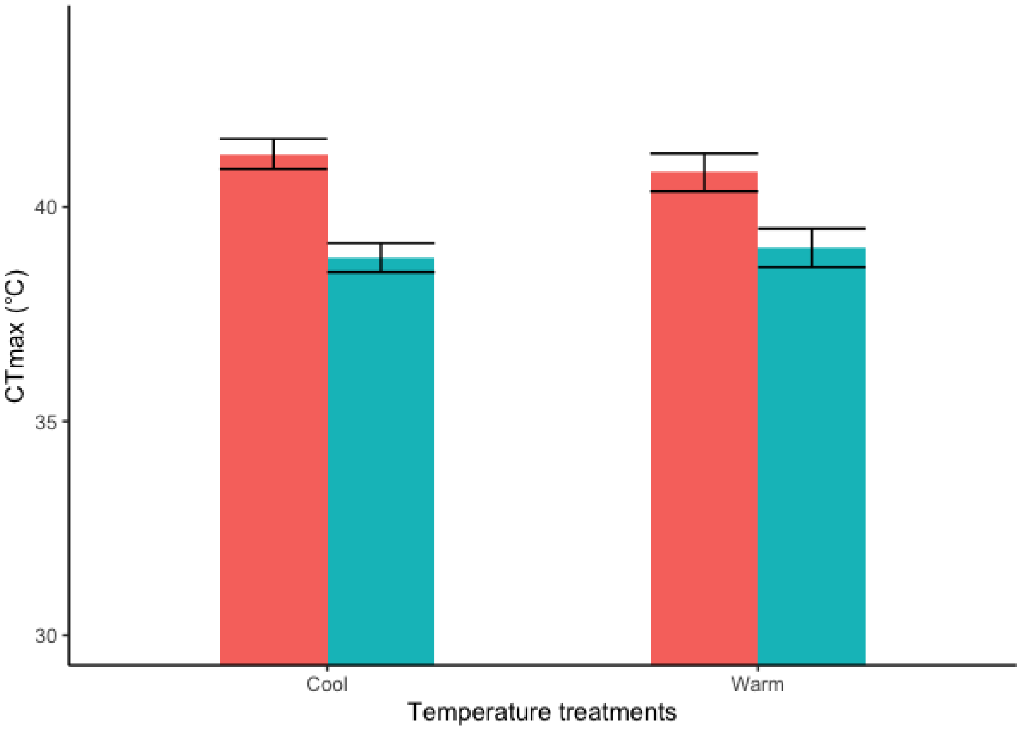

Figure 10. *Mean CTmax of* C. pictus *males from Northern (Red) and Southern (Blue) populations that were kept in warm and cool treatments over a 3-month period. Error bars represent ±1 SE. The northern population had higher Ctmax regardless of temperature treatment.*

2.4. Discussion

This study investigated metabolic and morphological differences between two populations of *C. pictus* from different thermal environments. Males from the hotter natal environment were larger in body size (mass and length), had higher metabolic rates at high temperatures, higher CTmax, basked more and invested more in reproduction (GSI) independent of body condition. The acclimation temperature treatment affected basking behaviour, and SVL growth, with lizards in the cool treatment growing in length considerably more than in the warm treatment. Thus, these results are suggestive of thermal adaptation within both populations of *C. pictus*. However, increased SVL growth (%) in the cool treatment contrasts with current evidence in ectothermic growth and instead highlighting the role of phenotypic plasticity in determining growth in the species.

2.4.1 Temperature clines in morphology

A negative temperature-body size cline is observed in many squamates (lizards and snakes) (Ashton and Feldman 2003). Although there are various hypotheses to explain such phenomena, the maintenance of preferred body temperature is one possible explanation for negative temperature-body size clines in this species. Increases in the surface area to volume ratio of an organism allows for more rapid heating and cooling, generating selection for smaller body sizes in cooler climates for reptiles, which enables them to warm relatively quickly (Cowles 1945; Bogert 1949; Stevenson 1985). In warmer areas, where ambient heat is more readily available, selection on thermoregulatory abilities is lessened, allowing ectotherms to reach significantly larger sizes and attain the many benefits associated with it such as; advantages in intraspecific competition (Martin *et al.* 2019), and investment in reproduction (Prado and Haddad 2005). Temperature-body size clines may also explain the contrasting reproductive investment strategies between the populations in this study (Perrin 1998).

Larger body size of the Northern *C. pictus* population may allow increased investment in reproduction, with GSI being three times higher than the Southern population. Additionally, GSI in the northern population was primarily independent from body condition, whilst in the Southern population, it was positively condition-dependant. Decreased testes size and condition dependant investment in reproduction is indicative of a population evolved in an environment that is sub-optimal in terms of resource availability and thermal environment (Angilletta and Sears 2000). A trade-off occurs between growth and reproduction and forces investment in reproduction to be a "luxury", which only individuals with high BCI may achieve effectively (Todd 2008; Uller *et al.* 2010). Alternatively, this discrepancy in GSI may be the result of male-male competition within both populations. Higher GSI correlates to high population densities in which competition for females is fierce and larger testes and sperm competition can increase chances of competitive fertilisation success (Kahrl *et al.* 2016). An example of this is testes size variation in the Cane toad invasion in Australia (*Rhinella marina*) (Friesen and Shine 2019). As population densities in this species are lessened at the edge of the invasion front, males experience considerably less sperm competition and thus have smaller testes (Friesen and Shine 2019). However, no published information on population densities and differences in resource availability is available for the two populations. Still, there is observational evidence that the northern population is less densely populated, potentially discrediting the population density GSI hypothesis (C.R. Friesen, unpublished observation). Without concrete evidence, inferences are limited and provide a future avenue of investigation. Regardless, these results demonstrate geographic variation in reproductive and somatic investment within this species.

2.4.2 Plasticity of growth

Surprisingly, in our study, SVL growth (%) was significantly higher for animals in the cool treatment, regardless of population. These results do not support our predictions that growth would increase as a function of temperature via increased metabolic activity (full review see (Angilletta *et al.* 2004)). However, a variety of explanation could account for temperature dependant growth rate seen in this experiment. A strong relationship between annual growth and annual activity time is observed in a variety of lizard species (Adolph and Porter 1993; Ferguson and Talent 1993; Flouris and Piantoni 2015). Therefore, when in the cool treatment, lizards may bask more throughout the day and be considerably more activity, evident via the marked increase in basking observed in the cool treatment. However, these results suggest another possible paradox; northern animals are larger, even though they originate from a warmer environment. Due to the positive correlation between SVL growth and $BCI_{initial}$, alongside the lizards being sexually mature, it is plausible the majority of growth was completed before reaching the laboratory (Dumas and France 2008). However, growth rates as a percentage are comparable between populations in captivity, so we suggest growth rates may be greatly enhanced during late autumn or early spring due to the increased temperatures and food availability to the Northern population (Metcalfe and Monaghan 2003). As there were only two temperature treatments to compare between, determining whether the cool treatment enhanced growth or the warm treatment suppressed growth is difficult. Therefore, for future studies, the addition of control temperature treatment, intermediate to the warm and cool treatments, would increase understanding of the relative effects of temperature on growth within this species (Atkinson 1996). Therefore, these results suggest plastic growth in response to temperature and indicate potential underlying differences in energetics budgets between the populations.

2.4.3 Population-specific basking behaviour

A simple interpretation of the basking patterns is that the Northern population, due to increased ambient temperatures in the source population, engage in more thermophilic (Basking) behaviour than the Southern population. However, cooler conditions may force individuals to bask more (Carter et al. 2010) and subsequently alter growth rate (Adolph and Porter 1993; Ferguson and Talent 1993; Flouris and Piantoni 2015). Underlying metabolic differences are a significant factor in driving in ectotherm basking behaviour (Stapley 2006). Individuals and species with higher metabolic needs demonstrate increased levels of basking behaviour (Mell et al. 2016). In Ballen *et al.* (2012) increased levels of basking in *C. pictus* led to increased activity levels and consequently, metabolic activity, potentially indicating that Northern populations have increased metabolic activity. On the other hand, *C. pictus* individuals have also been known to seek shade in the heat of the day (Melville and Schulte II 2001), a behaviour congruent to that observed throughout the acclimation period in both the Southern population, and to a lesser extent the Northern population in our study.

2.4.5 Population-specific metabolism

Population differences in metabolic performance curves, such as those found in this study, have been documented in a wide range of ectotherms (Bennett 1982; Sears 2005; Noyola *et al.* 2013; Hu *et al.* 2019). In many species, population differences in msRMR are believed to be adaptive and shift the population-specific Topt to suit the environment (Schulte *et al.* 2011). Shifts in Topt from thermal acclimation result in increased efficiency in utilising environmental heat, decreasing the amount of time needed to attain body temperature via thermoregulation and weakening selection on thermoregulatory traits (Angilletta *et al.* 2004). Improved efficiency results in increased energy availability which can increase; foraging time (Norberg 1977), reproductive investment (Angilletta and Sears 2000) and somatic investment (Lester *et al.* 2004), all of which can significantly enhance organismal fitness. A higher metabolic rate comes at a trade-off. When food resources are scarce, an increased RMR becomes a significant burden and can lead to individuals starving and, thus, large population diebacks may occur without sufficient phenotypic flexibility in RMR (Metcalfe and Monaghan 2003). Additionally, the physiological costs of enhanced metabolic activity, such as increasing reactive oxygen species production, may be a trade-off experienced by the Northern population and thus provides warrants future investigation (Smith *et al.* 2016). Our proposed shift of Topt is further supported via the Northern populations higher CTmax, potentially indicating the Northern population has an increased ability to deal with high temperatures (Addo-Bediako *et al.* 2000). Unfortunately, in the current experiment, temperatures during metabolic measurements were not raised high enough (> 40 °C) to report population changes in Topt accurately, which would be indicated by a significant drop in RMR with increased temperature (Angilletta 2009) as we began to see in the Southern population. However, the Northern Population is likely has a higher Topt as the Southern population's thermal performance curve flattens considerably after 30 degrees whilst increases can be seen in the Northern population. Therefore, we suggest that population-specific msRMR and thermal adaptations occur within *C. pictus due to* contrasting thermal environments and energetic requirements.

RMR is relatively understudied in polymorphic species. In polymorphic cichlids, the most dominant morph had the lowest metabolic rate, attributed to increased metabolic efficiency (Dijkstra *et al.* 2016). Morph-specific metabolism documented in another population of *C. pictus*, with temperature regimes intermediate to the two populations used in this study, was not found in this study. In Friesen *et al.* (2017a) males with bibs had a significantly higher metabolic rate at 36 °C, suggesting underlying differences in the behavioural strategies' energetic requirements. However, in this study, Bibbed males of both populations have similar msRMR to non-Bibbed males. Bibs are much more prevalent in the Southern population, and msRMR is lower, making Bib and Population confounded, potentially leading to type II errors. Thus, for future studies, and even selection of bibbed and non-bibbed males per population and greater sample size would be beneficial to increase statistical power.

The acclimation treatment had minimal impact on msRMR in both populations. Although the measurement of msRMR can correlate with metabolic capacity, it is not entirely reflective (Gomes *et al.* 2004). Resting MR is a measure of the ‘cost of living’ (Hulbert and Else 2000) and thus may be unsuited to capture acclimation responses, as selection should minimise RMR and maximise metabolic capacity. Hence, although informative, it may be inaccurate to use RMR as a measure of acclimation or adaptive response to environmental change (Rogers *et al.* 2007). Alternatively, employing mitochondrial oxygen metabolism measures alongside the measurement of metabolic enzymes such as lactate dehydrogenase, cytochrome c, and citrate synthase over a range of temperatures may allow for a deeper understanding of the thermal effects of acclimation on metabolism (Rogers *et al.* 2007). Enhanced population-specific acclimation responses would allow *C. pictus* to have increased reproductive success in thermally varied environments, thus expanding their ecological niche (Kearney and Porter 2004). Based on our results, *C. pictus* may have limited phenotypic plasticity to alter msRMR post-maturity, and therefore msRMR may be determined in ontogeny, or is a heritable trait. However, a common garden experiment is required to test this hypothesis fully.

Conclusion

The Northern population, sourced in significantly warmer conditions, demonstrated increased metabolic function compared to the cool adapted southern population, indicative of thermal adaptation. These thermal adaptations may provide increase metabolic scope for early developmental somatic growth and enable the Northern population to attain significantly larger sizes and invest more in reproduction. However, post-maturity growth measured in the lab did not differ between populations and was instead plastic to temperature treatments with individuals growing considerably more in the cool treatment, possibly due to increased activity. Therefore, natal thermal environment, thermal adaptation and phenotypic plasticity all play a critical role in determining variation of life-history traits within *C. pictus*.

Chapter 3:

Thermal effects on telomere length in a colour polymorphic ectotherm, the role of thermal geographic variation.

Acknowledgments:
All animal husbandry carried out by Dr. Christopher Friesen and Sandra Chatham.
qPCR carried out by Daniel Ritchie.
All data and R code is available upon request.

Abstract

Telomere dynamics in wild ectothermic populations is underexplored compared to their endothermic counterparts. Telomeres are non-coding ‘caps’ on the end of chromosomes, and their link to organismal and cellular self-maintenance may provide much-needed insight into the ecological evolution and development of life-history traits that shape population structure. The metabolic and life-history traits of ectothermic organisms are closely linked to their thermal environment. Increased temperatures are predicted to decrease telomere length and alter life-history traits. However, the utility of telomere length as a biomarker of stress and ageing associated with investment trade-offs among reproductive tactics is underexplored, especially within the context of geographic variation in colour polymorphic species. To address these issues, we assessed telomere length in warm and cool adapted populations of lizard, *Ctenophorus pictus*, which is colour polymorphic in the expression of a yellow throat patch, or “bib”. Lizards from both populations were acclimated to either warm or cool temperatures for three months. Telomere length was measured (using qPCR) in blood samples taken at the time of capture, early in acclimation, and at the end of acclimation to temperature treatments. We found that in the wild, non-bibbed males in the cool adapted population had significantly shorter telomeres than bibbed males, whilst in the warm adapted population, there was no difference. This result highlights the potential for divergence in the role of polymorphisms across populations. After the acclimation treatment, males from the cold-adapted population in the cool treatment had significantly shorter telomeres than any other group, regardless of Bib. These results contrasted with our predictions, highlighting the complex and counterintuitive relationship between environmental stressors, metabolic rate and telomere dynamics.

3.1. Introduction

Telomeres are repeating nucleotide motif (TTAGGG) at the end of chromosomes and play many crucial roles in cellular function and genome stability (Monaghan and Haussmann 2006). When DNA is replicated, telomeres shorten, providing a functional limit to how many times a cell can proliferate (Kawanishi and Oikawa 2004). Additionally, reactive oxygen species (ROS) produced in ATP formation can decrease telomere length due to ROS's high reactivity with the guanine-rich telomere sequences, i.e., GGG (Von Zglinicki 2002). There is a negative correlation between age and telomere length due to repeated cellular replication and persistent oxidative stress (particularly in endotherms; Monaghan and Haussmann 2006; López-Otín *et al.* 2013). Decreased telomere length can trigger cellular senescence and influence many key life-history traits in organisms (Aubert and Lansdorp 2008). Therefore, telomeres can be utilised as a metric of self-maintenance and biological marker of stress and ageing within individuals, with widespread implications for understanding ecological and evolutionary processes (Olsson *et al.* 2018a). Most investigations into telomere dynamics (attrition and elongation over time) have been conducted on endotherms, primarily humans and laboratory animals (e.g. reviews (e.g. Reviews; Gomes *et al.* 2011; Ingles and Deakin 2016). Our understanding of telomere dynamics in wild populations of ectotherms is burgeoning (Olsson *et al.* 2018a). Thus, understanding the interaction of environmental factors such as temperature on telomere dynamics in wild populations may explain variation in fitness across individuals and populations (Dugdale and Richardson 2018; Fitzpatrick *et al.* 2019).

The interaction of temperature, metabolism, and telomere dynamics over a species' geographic range will inherently depend on local adaptation to contrasting thermal conditions within the species (Kawecki and Ebert 2004). Selection within populations should optimise metabolic and cellular performance to improve fitness under local conditions (Angilletta 2009). Selection for thermal adaptation should be strong in ectotherms as their metabolic rate is intrinsically linked with ambient temperature (Angilletta 2009). Locally adapted ectothermic populations may physiologically respond differently to altered thermal conditions through changes in thermal optima and reaction norms of various metabolic processes (Monaghan *et al.* 2009). For example, as metabolic rate and ROS production are linked with temperature (Monaghan and Ozanne 2018), populations adapted to distinct climates may demonstrate contrasting telomere responses (Monaghan and Haussmann 2006;

Olsson *et al.* 2018a; Olsson *et al.* 2018b). Therefore, variation of temperature across the range of a species, through altering metabolic function, may influence telomere dynamics' and subsequent effects on life-history traits (Fitzpatrick *et al.* 2019). However, the extent of research conducted is limited, and the co-variation of each factor in the relationship is unclear, particularly as variation between individuals in telomere length is large.

Pace-off life (POL) theory suggests that organisms living a 'faster' life have enhanced growth rates, increased metabolic rate and reproductive output at the expense of decreased longevity than individuals with a slower POL (Ricklefs and Wikelski 2002; Jimenez *et al.* 2014; Dammhahn *et al.* 2018). As these suites of life-history traits evolve over the landscape, the role of environmental factors such as temperature in influencing this relationship is influential (Angilletta and Dunham 2003). In endotherms, a 'faster' POL is typically associated with cooler environments (Williams *et al.* 2010). In ectotherms, faster POL is associated with warmer areas (Pörtner 2002b), potentially due to increased metabolic rate and increased activity times experienced by both groups at those temperatures (Adolph and Porter 1993; Schmidt-Nielsen 1997; Nespolo *et al.* 2011). The link between a 'fast' paced life and decreased longevity has traditionally been attributed to elevated metabolic rate, increasing ROS production and subsequently telomere erosion (Monaghan and Haussmann 2006; Pauliny *et al.* 2015). Thus, temperature appears to be a crucial link between ROS production and metabolism and telomere dynamics and seems to hold for mammals and birds, with lower temperatures increasing metabolic rate and telomere erosion (Monaghan 2014). However, studies of ectotherms have demonstrated both increases and decreases of telomere length in response to thermal stress (Olsson *et al.* 2018a). The telomere dynamics across and along thermal clines has the potential to drive or reflect variation in life-history traits across populations (Dupoué *et al.* 2017; Dugdale and Richardson 2018; Ibáñez-Álamo *et al.* 2018)

The potential benefits and costs of maintaining long telomeres provide a classic life-history trade-off to understand individual fitness variation across populations, individuals, and morph types (Monaghan and Ozanne 2018). The existence of polymorphisms occurs when different phenotypes exist over evolutionary timescales within the same population (Stuart-Fox *et al.* 2021). If one morph has a specific fitness advantage over another, then that gene will fixate and become the only dominant phenotype. Thus, each morph must have a fitness advantage over another for a balanced polymorphism to occur. An example of frequency dependant selection is evident in the distinct pre-copulatory reproductive strategies of the Tree lizard (*Urosaurus ornatus*). The blue morph is dominant and defends more territory, the orange morph is non-territorial and is nomadic and generalist feeder when conditions are unfavourable whilst the yellow morph sneak’s copulations around territory of the blue males (Moore *et al.* 1998; Lattanzio and Miles 2016). Thus, each morph has a specific advantage over the other, and each morph's persists throughout the population (Lattanzio and Miles 2016). Telomere dynamics provides another mechanism by which morph-specific fitness advantages can be accrued.

For example, the Australian painted dragon (*Ctenophorus pictus*) is colour-polymorphic in head-colour (red, orange, blue and yellow) and yellow gular patch (bibbed or non-bibbed) (Olsson *et al.* 2007b; Healey and Olsson 2009). Telomere dynamics in one population in central NSW, reflect the different reproductive strategies between morphs (Rollings *et al.* 2017). Redheaded males have significantly shorter telomeres (Ballen *et al.* 2012; Rollings *et al.* 2017), attributed to a cost of higher testosterone levels (Olsson *et al.* 2007a) and increased territorial defence (Healey *et al.* 2007), whilst yellow-headed males had longer telomeres due to decreased testosterone and employing a less metabolically costly ‘sneaking’ reproductive strategy (Friesen *et al.* 2017a), out-competing red males in sperm competition trials (Olsson *et al.* 2009b). Additionally, the bib polymorphism consists of a gular throat patch made up of carotenoids, a potential antioxidant (Olsson *et al.* 2008b; Olsson *et al.* 2013). Colour is costly to maintain in terms of telomere attrition (Giraudeau *et al.* 2016) and must be protected against oxidative assaults (Olsson *et al.* 2012b), which varies among morphs (Friesen *et al.* 2017b). Males with bibs were found to experience significant body condition loss due to mate defence against non-bibbed males (Healey and Olsson 2009) as well as shorter telomeres than bibbed males (Rollings *et al.* 2017). Attributed to higher metabolic rate (Friesen *et al.* 2021), activity levels (Olsson *et al.* 2009a), lower endogenous antioxidants (Friesen *et al.* 2019) and higher levels of ROS (Friesen *et al.* 2021) a cellular maintenance cost of short-term reproductive success. However, all studies mentioned above in *C. pictus* have been conducted on the same population, despite this species occupying an extensive geographic range. The role of polymorphisms in separate populations is unknown, and populations may have contrasting thermal adaptions. These facts allow for a two-stage investigation into telomere dynamics across a temperature and aridity cline. Firstly, to assess the relative telomere length of individuals of different polymorphisms within their natural habit and secondly by thermally acclimating individuals from those populations in a common garden-style experiment to assess population-specific telomere responses to thermal treatments. To evaluate these hypotheses, males from a northern (warm adapted) and southern (cool adapted) population were acclimated to a warm and cool temperature treatment for 3-months. Telomere length was quantified from blood samples in the field, at the first time in the lab and the end of the three-month acclimation. We predict that there will be population-specific relationships between morph type and telomere length in the wild, with bibbed males having shorter telomeres than non-bibbed males (Rollings *et al.* 2017). Additionally, after acclimation, we expect that telomere length will be shortened in the warm treatment due to increased metabolic function associated with increased ambient temperature in ectotherms (Fitzpatrick *et al.* 2019). These effects will be most pronounced for the southern population due to temperatures being furthest away from their theoretical thermal optimum.

3.2. Methods

3.2.1. Animal collection, housing and temperature treatments

The Australian Painted Dragon lizard is a small agamid lizard endemic to south-central Australia. Mature (>9-month old Olsson *et al.* 2007b)) lizards were captured via hand or lasso in October 2019 from two discrete sites with hypothetically zero gene flow between them due to habitat fragmentation, Muloorina station (N = 20; 29°14'; 137°54') to the north, and Innes National Park at the tip of the Yorke Peninsula (N = 20; 35°14'; 136°54') to the south, hereafter called the North and South population (*Figure 11*). These sites represent arid and coastal environments due to significant differences in average daily temperature variation (high-low) and inter-season variation (*Figure 12*). Additionally, large differences in average annual rainfall with average yearly rainfall approximately three times lower at the northern site (350 mm vs 125 mm annually) (Bureau of Meteorology*).* As such, the northern site is classed as markedly hotter and more variable than the southern site.

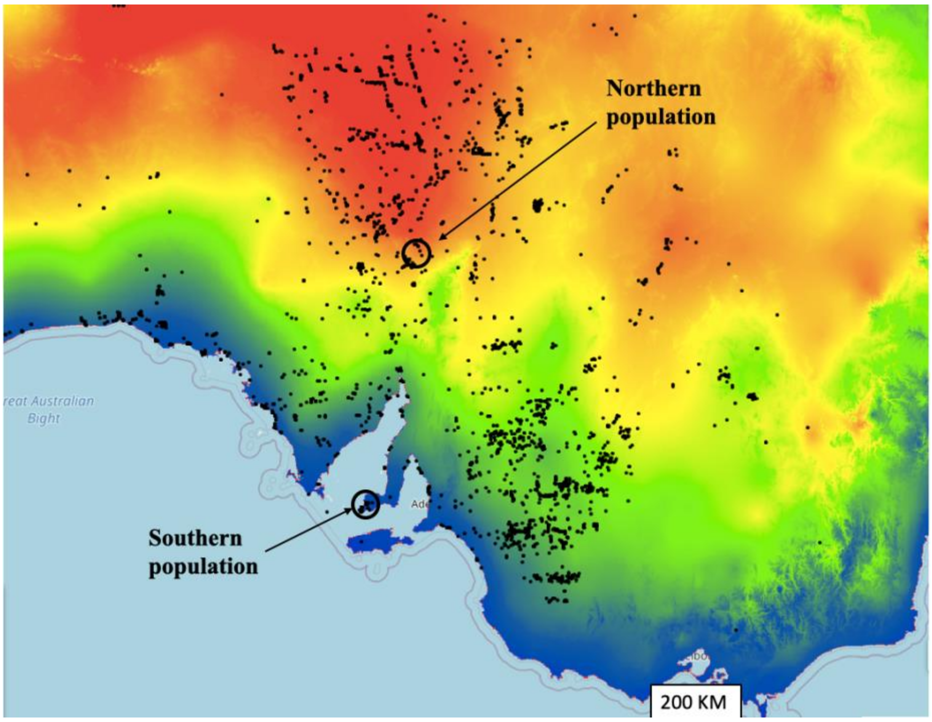

Figure 11. *Map of field sites in which populations of* C. pictus *were sourced; Muloorina station (Northern population) (29°14'; 137°54'), and Innes National Park (Southern population) (35°14'; 136°54'. Black dots indicate records of* C. pictus *from Atlas of Living Australia (https://www.ala.org.au/). Colour shading in the map indicates temperature/aridity (orange: high aridity, and blue: low aridity).*

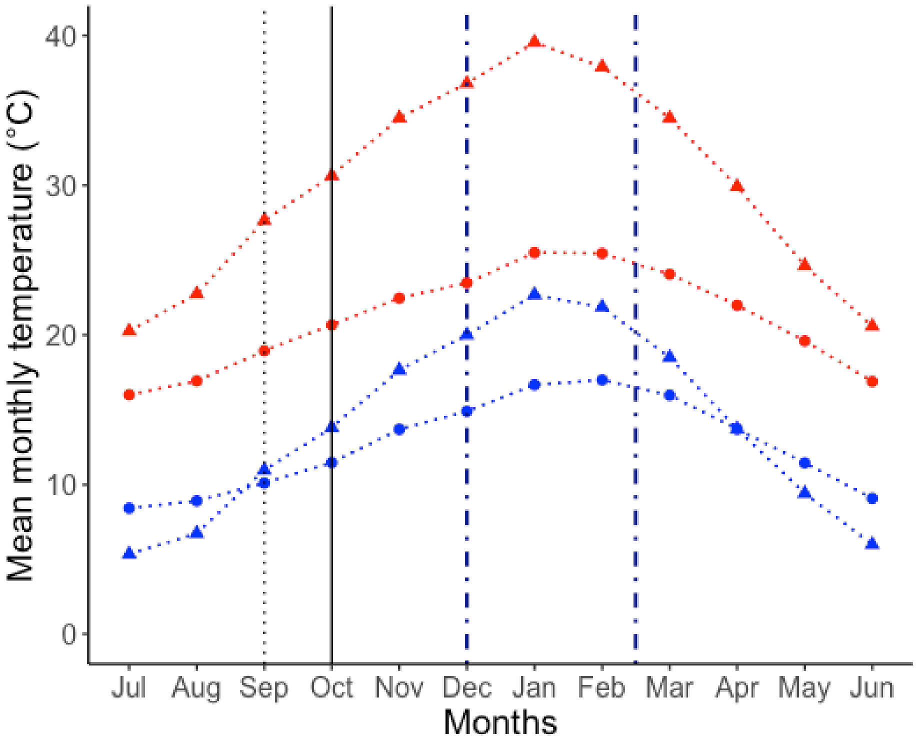

Figure 12. *Average daily High (red dotted line) and Low (blue dotted-line) temperatures for each month over the years 1996-2015 for the two locations; Northern population (Triangles) data from Marree, SA weather station (60 km from the field site). Southern population (Circles) data from Stenhouse Bay, SA weather station (20 km from the field site). Vertical lines indicate the presumed beginning of breeding season for the Northern population (dotted line) and Southern population (solid line). Vertical dot-dash lines indicate study period. Climate data sourced from the Bureau of Meteorology.*

Animals were sexed, scored for bib presence or absence, assigned ID, body size in terms of mass (±1 mg) and Snout-vent-length (SVL) (±0.5 mm) was measured and an initial blood sample was taken using a capillary tube and gently perforating the *vena angularis* (in the corner of the mouth). Lizards were transported to the University of Wollongong and housed individually in opaque 88L tubs (400 x 400 x 620 mm) with sandy substrate, a hide, and a heat lamp for thermoregulation. Males from each population were randomly allocated two separate rooms (Cool room N = 20, Warm room N = 20). The ambient baseline temperature control system in both rooms were set to 15 C°. One room had 40 W heat lamps, and the other had 60W heat lamps, which generated different temperatures within the lizard's enclosures and affected the ambient temperatures in each room during the day (Warm room mean = 24.7 °C, se = 0.1, Cool room mean = 20.4 °C, se = 0.1) and thus termed Warm and Cool treatments hereafter. Maximum mean temperatures directly under the heat lamps within the enclosures matched average maximum temperatures at both the Northern and Southern populations, (Max mean tub temperature warm room = 42.5 °C, se = 2.2), (Max mean tub temperature cool room = 28.5 °C, se =1.5, *Figure 1*). The lizards were kept in these temperature treatments for three months. The UV-B and ambient lights in each room were set to a 10 h:14 h L: D cycle, and heat lamps were assigned to a 7 h:17 h on: off regime. The animals were fed four mealworms dusted with calcium and pre-formulated multivitamin powder and misted with water every second day. Animals were collected under a scientific research license issued by South Australia Department of Water and Environment permit (A26776-1-4), and the collection and experiments were conducted in accordance with research authority granted by University of Wollongong Animal Ethics Committee (AE1907A04).

3.2.2 Experimental methods

3.2.2.1 DNA extractions

DNA was purified from blood samples taken in the field, when first in the lab and at the end of the experimental period. A DNeasy Blood and Tissue kit (Qiagen, Australia) was used for the extractions according to the manufactures instructions. The blood was digested with proteinase Kinase for 15 minutes at 56 °C. Subsequently, DNA concentrations and purity was assessed in duplicate using a Nanodrop (Thermo Fisher Scientific). All but six samples had concentrations above 10ng/mL and a 260/280 ratio in-between 1.7-1.9, which indicates pure DNA, thus final sample size between populations and temperature was (October, North/warm N = 9, South/warm N = 9, North/warm N = 10, South/cool N = 9) , (February North/warm N = 9, South/warm N = 8, North/warm N = 10, South/cool N = 10). All DNA samples were diluted to 10 ng/mL concentration and frozen at -20°C until use.

3.2.2.2 Quantifying relative telomere length

Relative telomere length (measured against the non-variable copy number reference gene 18S) was determined using real-time quantitative PCR (qPCR). We used QuantiFast SYBR green master mix containing Taq polymerase and SYBR green dye in the Rotor-gene 6000 thermocycler (Qiagen, Australia) using previously published protocols (Rollings *et al.* 2017). The primers used for telomeres were Telb1 (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Telb2 (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′). The gene 18s (Primer sequence18S-F (5′-GAGGTGAAATTCTTGGACCGG-3′) and 18S-R (5′-CGAACCTCCGACTTTCGTTCT-3′)) was utilised as the reference gene. Within each run, all samples of an individual at each time point were run in quadruplicate to ensure minimal intra-individual variation, and each run consisted of either the Telb or 18s primers. The initial taq activation step was 95°C for 10 minutes, and subsequently 40 cycles were completed at 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s with a melt curve being generated after each run from 60° to 95 °C to confirm no non-specific product amplification. Reactions had a final volume of 20µl, consisting of 20ng of DNA at 10 ng/ µl, forward and reverse primers at 250 nM, 4 µl of molecular grade H_2O and 10 µl of SensiMix SYBR green master mix. Each run contained a no template DNA control in quadruplicate to confirm no contamination, additionally a “golden sample” containing DNA of four individuals from each treatment group was run in quadruplicate to monitor for inter-run variation. The ‘golden sample’ was also used to quantify the standard curves for both 18s and Telb, using a serial dilution from 1:1 to 1:32, ensuring consistent amplification of template across concentrations. Reactions were classified as consistent linear correlations (18s $R^2 = 0.968$, Telb $R^2 = 0.982$) (*Figure 13*). The efficiency of primer amplification was 1.28 for Telb and 0.94 for 18s. PCR data was analysed using the program LinregPCR, which calculates the starting concentration (N_0) of individual samples based on an amplicon's mean efficiency, baseline fluorescence, and log-linear threshold cycle values. Relative telomere length (rTL) was calculated using the starting concentrations (N_0) of telomere (T) and control gene (S) through the formula T/S. The mean inter-assay coefficients of variation (ICC) for qPCR runs for telb (n=8) (ICC = 0.748, 95% CI = 0.373, 0.879), 18S (n=8) (ICC = 0.985, 95% CI = 0.942, 0.994) and relatively telomere length (ICC = 0.675, 95% CI = 0.292, 0.859). The relatively low ICC values were accounted for qPCR run as a random factor in the analyses (see statistical methods below).

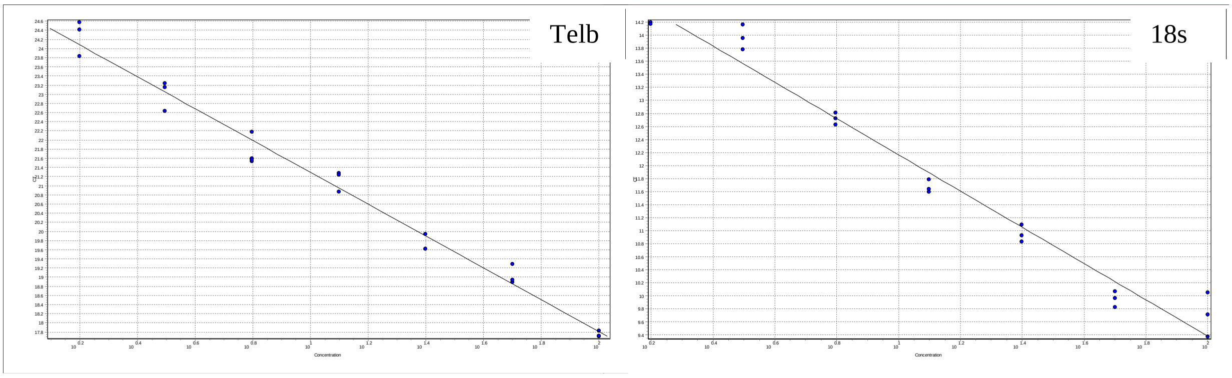

Figure 13. *Standard curves generated for both primers, Telb and 18s*

3.2.2.3 Morphological metrics and tissue collection

To quantify growth, measurements of mass (mg) and SVL (mm) were taken when the lizards first arrived in the lab and then in February at the conclusion of the experiment. After the 3-months, the lizards were euthanised to assess testes size alongside tissue samples being required of another experiment. The lizards were sedated via an injection of Zoletil (tiletamine/zolazepam). Once sufficiently sedated as indicated by loss of righting-response, they were euthanised with an overdose of sodium pentobarbitone and decapitated to ensure death prior to dissections. Subsequently, testes weight (±1 mg) was recorded. Testes mass was used as a robust proxy for male reproductive investment as post-copulatory selection regularly favours large testis size relative to body mass (Uller *et al.* 2010; Lüpold *et al.* 2020).

3.2.3 Statistical methods

3.2.3.1 Initial measurements in the field

All statistical analysis was conducted in R studio (RStudio Team (2019). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). To assess differences in relative telomere length), rTL data was first standardized (mean = 0; standard deviation = 1)

(Verhulst 2020) within each time point, in the field (rtLoctober), at the beginning of the experiment (rTLdecember) and the end of the experiment (rTLfeburary). A linear mixed effect model (LMM) using the R package "lme4" " (Bates et al. 2007) was constructed to assess rtLoctober variation in with population (North, South), Bib (No, Yes), the population by Bib interaction as the fixed effects and qPCR run as a random effect to account for high inter-assay variation. This yielded a significant effect of Bib. Thus, we explored the effect of Bib within each population by dropping population as a factor from the original model and analysed rtLoctober per population.

3.2.3.2 Body condition and telomere length at the onset of the experiment

Body condition index (BCI) was calculated as the standardised residuals (mean = 0; standard deviation = 1) from a linear regression of ln(body mass) as a function of ln(SVL) per population from the first measurement taken in the lab ($BCI_{initial}$). A linear regression between $BCI_{initial}$ and rTLdecember was constructed with population * $BCI_{initial}$ interaction to assess for initial population differences in the relationship.

3.2.3.3 Telomere length at the end of the experiment

A LMM was constructed with rTLfeburary as the response variable and population, temperature treatment, and interaction as fixed effects plus qPCR run as a random variable to account for inter-assay variation. Additionally, rTLoctober was included as a covariate to control telomere length at the beginning of the experiment, thus revealing the end rTL after controlling for the initial rTL thus excluding the potential effect of interstitial telomeres (Nussey *et al.* 2014; Bateson *et al.* 2019).

3.2.3.4 Body condition, growth and telomere length at the conclusion of the experiment

Body condition index (BCI) was calculated as the standardised residuals (mean = 0; standard deviation = 1) from a linear regression of ln(body mass) as a function of ln(SVL) per population at the conclusion of the experiment (BCI_{final}). A linear regression between BCI_{finall} and rTLfeburary was constructed with population * BCI_{final} interaction to assess for initial population differences in the relationship. To assess the effect of growth on telomere length, change in Snout-to-vent length (SVL) was calculated by subtracting SVL at the end of the experiment from SVL at the onset of the experiment, yielding SVLchange (mm). A linear regression between SVLchange and rTLfeburary was constructed with Bib and population included as factors, but neither was significant. However, temperature treatment was significant, and the treatments were regressed separately to explore the relationship between SVLchange and rtLfeburary further. Reproductive investment was evaluated by calculating the percentage of testes mass relative to body mass (testes mass/body mass x 100: Gonadal somatic index (GSI)). The relationship between GSI and rTLfeburary per population was assessed in a linear regression; GSI and rTLfeburary did not vary significantly between Bib or temperature treatment.

The results of all LMM's were assessed using a Type III, sums of squares (SS) ANOVA using Satterthwaite's method within the package "lmertest" (Kuznetsova et al. 2017). R^2 of LMM's were calculated using the R package "MuMIn" (Barton and Barton 2015). The significance level for all tests was $\alpha = 0.05$. Graphs were constructed using the R package "ggplot2" (Wickham 2016). R packages "tidyverse" (Wickham 2017) and "dplyr" (Wickham et al. 2015) were used for data handling and manipulation.

3.3 Results

3.3.1 Initial measurement in the field

Relative telomere length (rTL) in the field did not significantly differ between the northern (*n = 19)* and southern populations (*n = 18)* ($F_{1,29} = 0.248$, P = 0.622). Bib frequency differed between populations with the Southern population being 52% bibbed and the northern population being 38% bibbed. Opposite to predictions and previous work (Rollings *et al.* 2017), rTL was significantly shorter for non-bibbed individuals in the southern population (*n* = 8), than individuals with bibs (*n* = 10) ($F_{1,16} = 5.285$, P = 0.035). Whereas in the Northern population, there was no difference between bibbed (*n = 8*) and non-bibbed (*n = 11*) males ($F_{1,14} = 1.176$, P = 0.296) (*Table 4, Figure 14*). Initial body condition (BCI_{intial}) was not significantly associated with rTLDecember, nor was this influence by population ($F_{3,33} = 1.212$, P = 0.321).

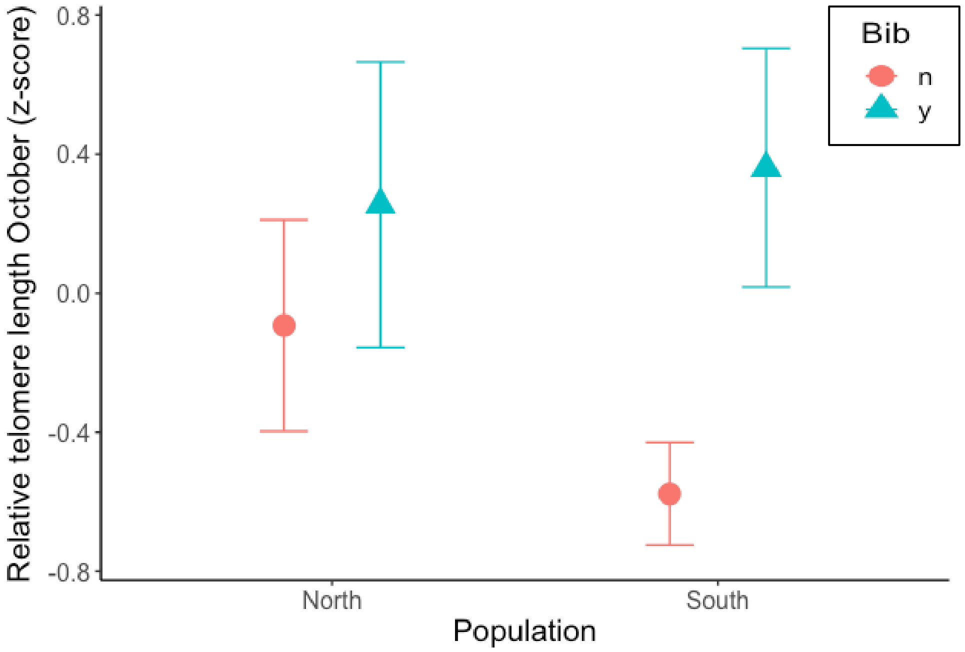

Figure 14. *Interaction plot of population and bib presence (blue triangle) or absence (red circle) in regards to relative telomere length (rTL) (z- score) at time of capture in* C. pictus. *All symbols are presented as the mean, and error bars indicate +- SE.*

Table 4. *Population-specific rTLOctober. Output of type III SS ANOVA from LMM using Satterthwaite's method. Bold text in the p-value column indicates significance at P ≤ 0.05.*

Southern population

	Model R^2	Sum Sq	Mean Sq	NumDF	DenDF	F value	P-value
Bib	0.237	3.906	3.906	1	16	5.285	**0.035**
Northern population							
Bib	0.041	0.659	0.659	1	14	1.176	0.296

3.3.2 Telomere length post acclimation

Relative telomere length (rTL) in February was significantly lower for the Southern population in the cool treatment (*n=8*) compared to the warm treatment (*n=9*). However, in the Northern population, there was no difference between warm (n=9) and cool treatments (*n=10*) (*Table 2, Figure. 15*). Telomere length in October as a covariate significantly predicted telomere length at the end of the experiment in February ($F_{1,29}$ = 4.681, P = 0.049).

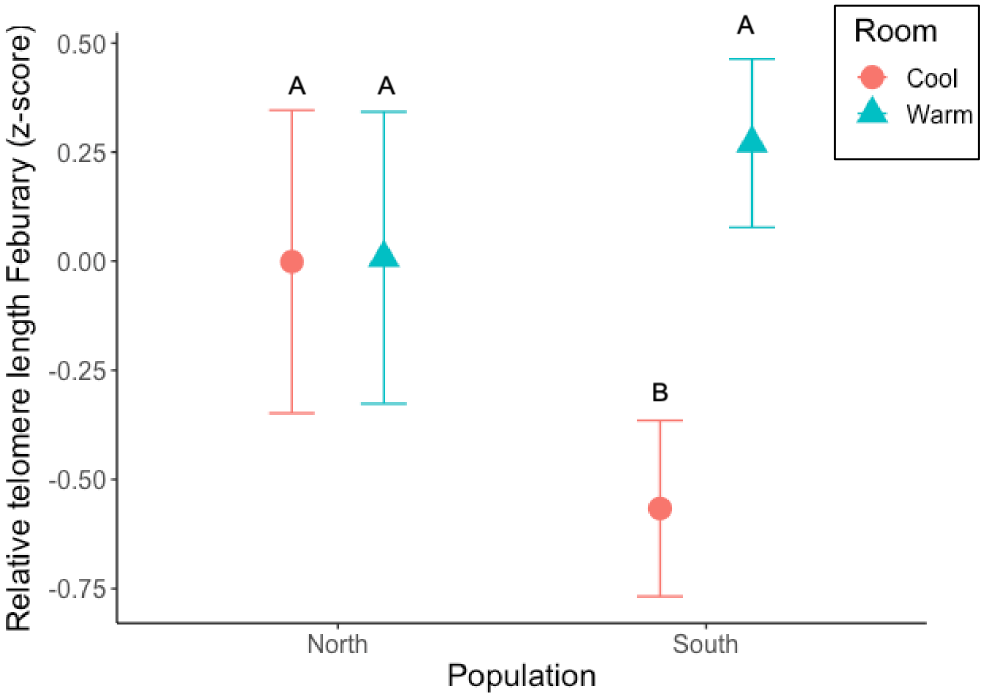

Figure 15. *Interaction plot of population and temperature treatment in* C. pictus *males in terms of relative telomere length in February (rTL) (z-score). All symbols are presented as the mean, and error bars indicate +-SE. Letters indicate significantly different means in pairwise comparisons (Tukey's method adjustment of alpha significance).*

Table 5. *rTL February with rTL December as covariate. Output of type III SS ANOVA from LMM using Satterthwaite's method. Bold text in the p-value column indicates significance at* $P \leq 0.05$.
Model R^2 =0.200

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P-value
Population	0.451	0.4528	1	24.697	0.610	0.442
Room	0.374	0.3740	1	29.620	0.504	0.483
Population*Room	3.131	3.4739	1	26.453	4.219	**0.039**
rTL October	3.474	3.4739	1	29.960	4.681	**0.049**

Temperature treatment, significantly affected the relationship between SVL change (mm) and rTLFebruary for the warm room ($F_{1,17}$ = 5.021, P = 0.0387, R^2 = 0.228), whilst the cool room had a non-significant relationship ($F_{1,16}$ = 0.1496, P = 0.704, R^2 = 0.009) (*Figure 16, Table 5*). Population did not significantly affect the relationship between GSI and rTLFeburary for either population ($F_{3,33}$ = 1.212, P = 0.321, R^2 = 0.099). Additionally, BCI_{final} was not effected rTLFeburary for either population ($F_{3,33}$ = 0.092, P = 0.964, R^2 = 0.092).

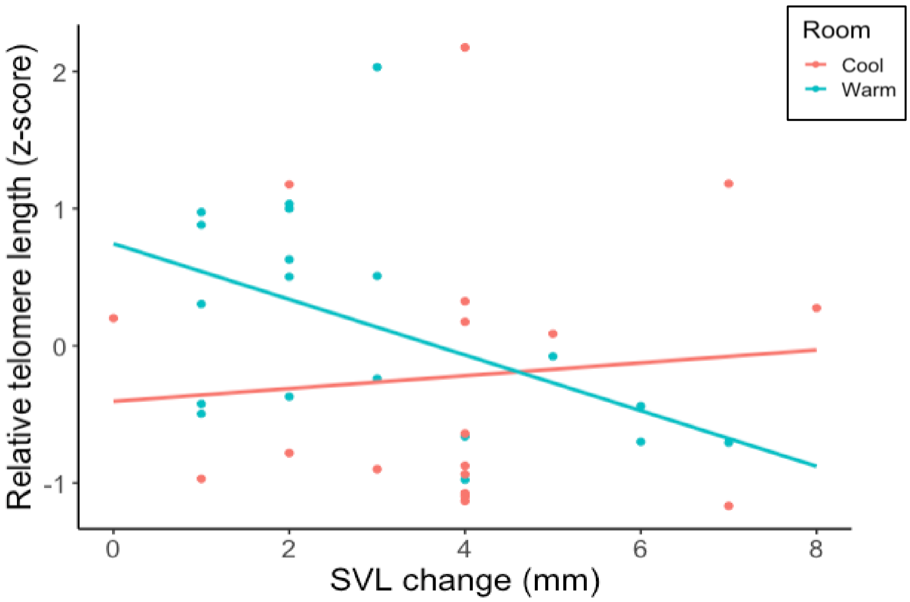

Figure 16*. Scatterplot showing contrasting correlations of treatment specific SVL (mm) and relative telomere length in February (z- score) across both populations of* C. pictus*, Cool (Red) and Warm (Blue). Coloured lines depicting treatment regression lines.*

3.4 Discussion

We investigated differences in telomere length between; populations, bib morphs and the effect of long-term thermal acclimation upon these groups. In the wild, non-bibbed males from the southern population had significantly shorter telomeres than bibbed male's and northern males from both morphs. By the end of the acclimation period, southern males in the cool room had significantly shorter telomeres than all other groups. Additionally, individuals in the warm treatment demonstrated a negative relationship between SVL growth and relative telomere length. These results highlight the differences in physiological costs associated with possessing sexual adornments across the geographic range of a *C. pictus*. We also found an interaction of thermal acclimation across populations in determining telomere dynamics, highlighting the potential costs of altering thermal regimes on ectothermic physiology.

3.4.1 Geographic variation in morph-specific self-maintenance

Morph-specific self-maintenance is intrinsically linked to the physiological costs associated with different morphs across various taxa (Karell *et al.* 2017; Rollings *et al.* 2017; Morosinotto *et al.* 2020). Non-bibbed males in the southern population with shorter telomeres contrast with other studies on *C. pictus,* which found that bibbed males had shorter telomeres (Rollings *et al.* 2017). The yellow gular patch in *C. pictus* is produced via a carotenoid deposit and is common in the scales of other yellow-coloured reptiles (Olsson *et al.* 2013). As carotenoids cannot be manufactured in the body, the intensity of pigments is dependent on both environmental availability and foraging efficacy (Steffen and McGraw 2009; Shawkey and D'Alba 2017), thus making their accumulation costly to maintain (Giraudeau *et al.* 2016). Due to this fact, the possession of a bib has been shown to require additional resources and produces trade-offs against key life-history traits like self-maintenance, growth and tissue repair (Olsson *et al.* 2008a; Rollings *et al.* 2017; Friesen *et al.* 2021). However, all previous studies on *C. pictus* have focused on a single population in Yathong, nature reserve NSW. Thus, our results indicate the role of bibs across the geographic range of *C. pictus,* in terms of pre-copulatory sexual selection, may be starkly different.

Pre-copulatory sexual selection works under two main principles, intrasexual and intersexual competition (Candolin 2000; Hunt *et al.* 2009). Intrasexual competition involves individuals of the same sex, typically males competing against each other for limited mating options (Hunt *et al.* 2009). In *C. pictus,* intersexual competition exists between males, with individuals possessing bibs typically outcompeting non-bibbed male in the Yathong populations (McDiarmid *et al.* 2017). In unpublished work on the same lizards used in this study, it was found that bibbed males were more aggressive and more likely to win dyadic competitions than non-bibbed males, regardless of population (Chatham 2020 (unpublished)). Conversely, in intersexual competition trails, which involved female mate choice through the assessment of secondary sexual characteristics, bib presence in this case (Weaver *et al.* 2017). Females from the southern population demonstrated no preference towards either bib morph in female choice trails. However, there was a strong preference toward bibbed males in the northern population, even when bib colour was experimentally altered and despite the lower frequency of bibs in the population (Chatham 2020 (unpublished)).

When we assess these results in terms of telomere length in the wild, bibbed males in the Northern population appear to have no “handicap” in terms of the negative physiological costs associated with shorter telomeres whilst the trait also being heavily selected for in inter and intrasexual competition (Chatham 2020 (unpublished)). However, the infrequency of the bib phenotype within the population suggests negative density-dependent selection, in which rare traits carry a selective advantage (Roulin 2004). The potential fitness costs that keep bibbed males from occurring in ratios similar to non-bibbed males, such as post-copulatory selection on sperm performance and possible increased predation pressure due to increased visibility, is unexplored and would provide great insight into the role of bibs in the Northern population. In the southern population, the shorter telomeres in non-bibbed males may suggest possible adverse physiological costs alongside intersexual selection operating against non-bibs. However, intrasexual selection appears to favour neither bibbed or non-bibbed males. It is doubtful that the fitness advantages accrued by this phenomena are enough to explain the frequency of the bib polymorphism in the southern population. Thus, investigations into the pre-copulatory differences are another piece of the puzzle needed to understand the existence and maintenance of polymorphisms within the population. Therefore, these results highlight geographic variation in morph-specific self-maintenance and potentially indicates underlying differences in morph roles within and among populations.

3.4.2 Thermal acclimation response

Contrary to our predictions, relative telomere length at the end of the three-month acclimation was significantly shorter only for southern males in the cool room. Our predictions were based on the assumption that increased temperature would increase; metabolic rate (Gillooly *et al.* 2001), ROS production (Suzuki and Mittler 2006) and growth rate (Russell *et al.* 1996), all factors associated with increased telomere attrition (Von Zglinicki 2002). Thus, we expected that individuals kept in the warm treatment would experience telomere attrition. Additionally, we predicted this would be population-specific, with the southern population experiencing more significant telomere attrition than the northern population in the warm treatment and *vice-verse* in the cool treatment due to contrasting thermal adaptations. However, theoretically, the assumptions our predictions are based on are sound; empirically, there is contrasting evidence for each assumption.

Firstly, correlations between increased metabolism at warm acclimation temperatures and increased ROS production in ectotherms is limited. Studies such as (Bury *et al.* 2018) demonstrate that grass snakes (*Natrix natrix*) acclimated at warmer temperatures exhibit decreased ROS levels despite increased metabolic rate. One explanation for such phenomena is increased mitochondrial uncoupling occurring at higher metabolic rates and decreasing ROS production (Speakman *et al.* 2004). Mitochondrial uncoupling is any process in which electron transport is not used in ATP production and bypasses oxygen substrate, lowering membrane potential and decreasing ROS production (Mookerjee, Divakaruni et al. 2010). Thus, to assess for this possibility in future investigations, the use of high-resolution respirometry on isolated mitochondria may be a valuable tool in evaluating mitochondrial efficiency over a range of temperatures (Lanza and Nair 2010). If low levels of uncoupling occur at warmer temperatures, they may attenuate ROS production and subsequently limit their adverse effects on telomeres.

Secondly, the relationship between ROS production and telomere erosion has also been questioned (Fitzpatrick *et al.* 2019). Generally, in endotherms, this relationship has a solid empirical backing (Monaghan and Ozanne 2018). However, in various terrestrial ectotherms, including *C. pictus*, a strong negative relationship between ROS and telomere length has not been observed (Giraudeau *et al.* 2016; Ujvari *et al.* 2017; Fitzpatrick *et al.* 2019). Unfortunately, we were unable to assess the oxidative stress of *C. pictus* due to logistical reasons, so no such correlations could be evaluated. However, if such measurements were available, positive or, no correlations between ROS levels and telomere length would provide evidence of ROS independent telomere dynamics in ectotherms.

Finally, temperatures effect on growth rate and subsequent impact on telomere length has varied empirical evidence in vertebrate ectotherms (Monaghan and Ozanne 2018). As cell replication associated with growth and somatic maintenance is a significant component of telomere erosion it is widely considered a primary contributor to telomere dynamics (Aubert and Lansdorp 2008). In Friesen *et al.* (2021), *C. pictus* SVL growth was negatively correlated with telomere length. However, compensatory growth in brown trout juveniles was found to not affect telomere attrition (Näslund *et al.* 2015). In the current study, percentage SVL growth was, in fact, lower for the warm treatment, whilst percentage mass growth was not affected by temperature treatment in either population (Chapter 2). A possible explanation for this is that telomere length was negatively correlated with SVL growth (mm), but only in the warm treatment, suggesting that the costs associated with growth in the warm treatment were much higher than in the cool room, inhibiting growth. However, the stronger trend of shorter telomere length in the cool room seemingly clashes with this hypothesis. Conversely, increased basking activity levels within their enclosures may have negatively affected growth and telomere length. The cool treatment may have suppressed short term metabolic efficiency and forced individuals to become more active throughout the day, increasing growth, cell proliferation and subsequent telomere erosion, particularly for southern males (Adolph and Porter 1993; Ferguson and Talent 1993; Flouris and Piantoni 2015). Additionally, in the current study, food intake was not assessed and has been shown to have considerable impacts on telomere length (Friesen *et al.* 2021). Food consumption may potentially explain a high proportion of growth variation between temperature treatments whilst also allowing for increased metabolic function, without the typical negative physiological impacts on telomeres, characteristic in an energy-limited system (Friesen *et al.* 2021). Thus, food consumption is advised to be included in future studies. The factors mentioned above highlight the complex and, at times, counterintuitive interaction between temperature, metabolism, ROS production and telomere dynamics.

Geographic variation telomere response to temperature was found in this experiment and is the first of its kind documented in an ectothermic vertebrate. The southern population in the cool treatment was the only group to exhibit significantly shorter telomere lengths at the end of the experiment. This result was surprising due to the previous evidence of contrasting thermal adaptions between the populations regarding; size, resting metabolic rate and critical temperature points (Chapter 2). These results suggest that the southern population basked significantly more, and thus their energy budgets were conserved compared to if they were in an energy-limited system (Chapter 2) (Friesen *et al.* 2021). Therefore, increased activity provides the potential for the lower temperatures to decrease metabolic energy and thus limit antioxidants production, accelerating telomere erosion. However, in the northern population, which we predicted would experience telomere erosion in the cool treatment, this trend was not observed. A similar interaction between adaption, thermal acclimation and telomere dynamics was observed in spotted snow skink (*Niveoscincus ocellatus*) (Fitzpatrick *et al.* 2019). Males from cool adapted and warm adapted populations were subjected to warm and cool treatments. Lizards in the warm treatment had increased telomere length compared to the cool treatment, regardless of ROS levels and population. Evidently, the effect of thermal adaptation in determining telomere dynamics in ectotherms is in its nascent stages. More research into the interrelation of thermal adaptation, metabolic rate and telomere attrition is encouraged.

Variation in the thermal reaction norms of telomerase expression may be a significant component in explaining this experiment's results. Telomerase operates to lengthen short telomeres, thus increasing the limit of cell replication (Taylor and Delany 2000). Although in endotherms, telomerase production is limited to initial development, ectotherms can upregulate telomerase production throughout somatic growth and may be able to increase telomere length over the life of an individual (Hatakeyama *et al.* 2016). Little work has been done on the interaction between environmental stressors, enzymatic thermal performance curves and telomerase expression (Monaghan and Ozanne 2018). Thus, future work on the co-variation of telomerase expression and oxidative status in response to thermal stressors in whole animal studies may enable an increased understanding of complex interaction of temperature and telomeres in ectotherms.

Conclusion

To summarise, this study provides evidence for geographic variation in telomeric dynamics in response to thermal acclimation, one of the first of its kind in terrestrial vertebrates. We demonstrated that thermal acclimation can mediate telomere dynamics and that this occurs on a population-specific basis. Although this study represents a step toward understanding these complex effects, the exact mechanisms underpinning such phenomena are elusive. Additionally, contrasting telomere lengths from wild populations provides evidence for geographic variation in physiological processes underpinning polymorphisms and the potential roles they play in both populations. However, to fully understand the complexity of these phenomena, future studies are encouraged to investigate the temperature dependant role of ROS, telomerase expression and metabolism and how these traits may interact within a variety of populations and species.

Chapter 4: General conclusions

Geographic variation of physiological traits is an incredibly useful model to study a vast magnitude of ecological and evolutionary phenomena (Pörtner 2002b; Stillwell 2010). In its simplest form, geographic variation of traits is formed by divergent selective pressures operating on separate populations, creating different phenotypes (Gould and Johnston 1972). Geographic variation in thermal adaption is particularly strong for exothermic organisms due to strong links between their metabolic rate and ambient temperatures (Angilletta 2009). However, it has only been in recent years that variation in the biomolecular costs associated with sub-optimal temperatures potential has been thoughtfully investigated in ectotherms (Olsson *et al.* 2018a). These questions may be of particular importance in the face of large scale climate warming, which is predicted to alter the thermal environment of many ectothermic species (VanDerWal *et al.* 2013) (IPCC, 2014). This thesis aimed to investigate the role of temperature in influencing various thermal, biological traits in terms of thermal adaption, thermal acclimation, and potential biomolecular costs in two populations of a polymorphic dragon species.

Ctenophorus pictus is an ideal species to study variation in thermal biology, thermal acclimation and biomolecular costs, as they occur over a wide range of thermal environments and thus are exposed to various strengths of selective processes. Additionally, the possession of polymorphisms enable the investigation of thermal effects across various phenotypes within and between populations. A vast body of work has already been conducted on the species and has highlighted significant variation in many traits, attributed to the reproductive tactics of the polymorphisms (e.g. body condition (Healey and Olsson 2009) telomere dynamics (Rollings *et al.* 2017), metabolic rate (Friesen *et al.* 2021), activity levels (Olsson *et al.* 2009a), antioxidants (Friesen *et al.* 2019) and ROS (Friesen *et al.* 2021)). However, little work has been done on the geographic variation of these traits and potential underlying differences in the thermal biology of populations occupying contrasting thermal environments. As such, this study aimed to investigate broader questions of ectothermic response to temperature and expand the existing knowledge base of this species.

The findings of this thesis indicate the complex interaction between adaptive and plastic responses to thermal acclimation. Some traits, such as growth, were plastic for both populations to acclimation temperature. Other traits such as basking behaviour and telomere length reacted asymmetrically between populations and acclimation treatments. Whilst other traits responded solely on a population-specific basis (e.g. Body size, reproductive investment, metabolism and thermal critical points). Overall, these results suggest the determinants of thermal physiological traits across the range of *C. pictus* operate via the interaction of heritable or determinate traits and phenotypic plasticity.

When these results are compared to the existing literature, we find that many of the factors influenced by population have a large empirical backing and are indicative of thermal adaptation (e.g. Body size (Roff 1993), reproductive investment (Wikelski 2005), metabolism (Adolph and Porter 1993)and thermal critical points (Addo-Bediako *et al.* 2000)). Interestingly, the interaction of population and thermal acclimation in telomere length is the first of its kind in vertebrate ectotherms. The exact mechanisms controlling such an interaction remain elusive, with current theoretical predictions clashing with the results found in this study (i.e. cool adapted population experiencing shortened telomeres in cool treatment). Unfortunately, a lack of oxidative stress markers hampered the interpretation of variation in telomere length. Due to logistical constraints, three biomarkers of oxidative stress (Malondialdyhe a marker of oxidative stress upon lipids, Glutathione peroxidase and Superoxide dismutase, both primary endogenous antioxidants) were planned to be assayed in blood plasma samples taken at the end of the acclimation period. Their exclusion limits a wide range of interpretations regarding telomere length change. As such, they are highly advised for inclusion in future studies of this nature. Additionally, the inclusion of metrics such as mitochondrial efficiency, telomerase expression and the potential for temperature dependant expression of these metrics will significantly aid in delineating the relationship of temperature and telomere dynamics. However, increased basking time in the cool treatment may prove a valid explanation for such a phenomenon by increasing overall activity levels and accelerating cellular aging. If such results are repeatable across a variety of populations and species, then more concrete estimations of the biomolecular costs associated with altered thermal regimes can be made.

Expected differences within the bib polymorphism were not found in the two populations studied, apart from telomere length measurements in the field. The lack of distinction between bibbed and non-bibbed males may be attributed to the decreased bib ratios in the northern population, decreased statistical power, and Type II errors. Regardless, telomere length differences between southern bibbed and non-bibbed males in the wild contrasted with another population of *C. pictus* in which bibbed males have shorter telomeres (Rollings *et al.* 2017). These results potentially indicate the role of the bib polymorphism, and physiological costs associated with it may vary on a geographic basis. More research into the geographic variation of bib ratios across the range of *C. pictus* is needed, alongside a more detailed investigation into post-copulatory selection to understand the role of bibs fully. Moreover, quantifying the gene-flow between populations of *C. pictus* through genomics will allow a greater understanding of the relationship between potential source and sink populations and levels of relative adaption across the range of *Ctenophorus pictus*.

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