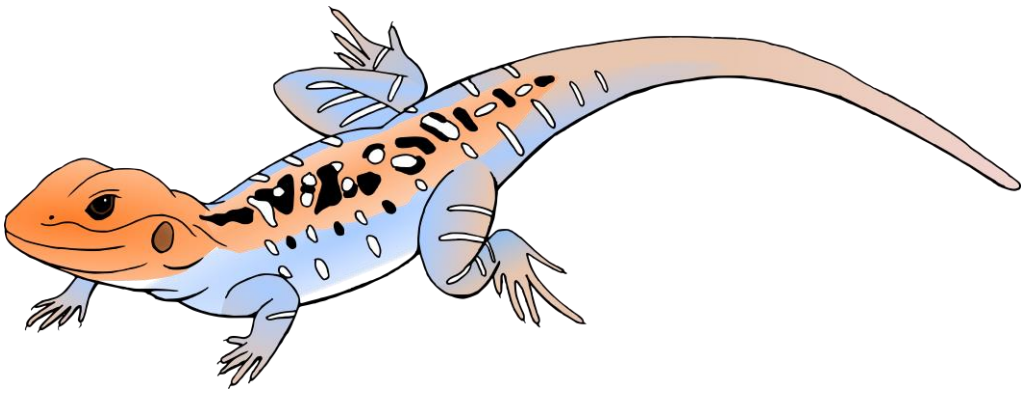
Variation in Telomere Dynamics with Life History Strategy in Polymorphic Reptiles, *Ctenophorus pictus* and *Thamnophis sirtalis parietalis*



A thesis submitted in fulfillment of the requirements for the degree of
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PREFACE

All data chapters of this thesis are presented as manuscripts that are either published in peer-reviewed journals, currently under consideration for publication, or in preparation for submission. As such, there is some repetition of background and methodology between the chapters.

All of the chapters within this thesis have been written with the guidance of my supervisors, Professor Richard Shine, Dr Camilla M Whittington, Dr Christopher R Friesen and former primary supervisor Professor Mats Olsson. Many co-authors have also contributed to the content of this thesis: Professor Mark Wilson, Dr Joanna Sudyka, Dr Mathieu Giraudeau, Dr Emily J Uhrig, Dr Randolph W Krohmer, Dr Heather L Waye, and Professor Robert T Mason. The individuals named above all contributed variously to the conception and design of experiments, collection of samples and field work, laboratory experiments and husbandry, data analysis and the editing of manuscripts. I designed experiments, carried out field work and animal husbandry, optimised and performed all of the qPCR analyses, performed data analyses, and wrote and edited all manuscript drafts. Cover illustrations are author's own.

AUTHORSHIP ATTRIBUTION STATEMENT

Chapter 2 of this thesis is submitted to Ecology and Evolution as "Sex-based differences in telomere length in somatic tissues of a reptile" with the following authorship: Rollings, N., Friesen, C.R., Whittington, C.M., Johansson, R., Shine, R., Olsson, M.
I designed the study, collected samples, performed the qPCR analysis, analysed the data, and wrote and edited the draft MS.

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I performed the qPCR analysis, analysed the data, and wrote and edited the draft MS.

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I designed the study, collected samples, performed the qPCR analysis, and wrote and edited the draft MS.

Chapter 5 of this thesis is published as Rollings, N., Uhrig, E.J., Krohmer, R.W., Waye, H.L., Mason, R.T., Olsson, M., Whittington, C.M., Friesen, C.R. (2017). Age-related sex differences in body condition and telomere dynamics of red-sided garter snakes. *Proc. Biol. Sci., 284*(1852), 9. doi:10.1098/rspb.2016.2146.

I performed the qPCR work, and wrote and edited the draft MS.

Chapter 6 of this thesis is submitted to the Journal of Experimental Zoology Part A as "Sperm telomere length correlates with growth rate in red-sided garter snakes, *Thamnophis sirtalis parietalis"* with the following authorship: Rollings, N., Waye, H.L., Krohmer, R.W., Uhrig, E.J., Mason, R.T., Olsson, M., Whittington, C.M., Friesen, C.R.
I performed the qPCR work, analysed the data, and wrote and edited the draft MS.

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.
I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.
In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Nicole Anne Rollings

Sydney, February 2019

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Professor Richard Shine

Sydney, February 2019

Dedicated to my dad, Jim Rollings (1952 – 2014).

I finished my Fh’d.

ACKNOWLEDGEMENTS

While I doubt that any PhD really ever goes exactly as planned, I suspect that between health issues and the movement of multiple supervisors my PhD went even less as planned than usual. Given how unconventional my PhD has been it seems fitting that I'm writing these acknowledgements months ahead of schedule. Regardless, due to the extensive support I've received I've managed to make it in the end. I must have, for you are reading this.

My thanks first go to my incredibly patient supervisors, who have been veritable fonts of knowledge. I've learned much from the lessons you have provided, intentional or otherwise. Firstly, **Mats Olsson**, thank you for introducing me to the world of telomeres which I find to be a source of endless fascination. While your departure from Usyd had suboptimal timing it certainly taught me to bounce when I hit the ground. **Chris Friesen**, your calm demeanour but dark sense of humour was always great company during the slightly maddening processes of sample collection. Your thoroughness with respect to manuscript writing and structure certainly helped me improve upon my previous approach of throwing words on paper and hoping for the best. **Camilla Whittington**, your intense attention to methodological detail has not only improved the quality of the research contained within this thesis, but will also stand me in very good stead when conducting assays in the future. Finally, **Rick Shine**, thank you for filling the suddenly vacant position of primary supervisor. I imagine accidentally becoming your student is an honour few can boast. Thank you, also, for obtaining emeritus status before leaving Usyd, allowing you to remain my primary supervisor (you were clearly playing the long game). Thank you all, as well, for your extensive feedback on all of my writing. Learning how to express concepts clearly and concisely will serve me well, regardless of where the future might take me.

I of course thank the many people involved in the **Olsson, Shine and Whittington-Thompson labs** who have offered both practical support and friendship over the years. I'll always remember the teamwork and camaraderie of Team Pictus (**Cissy Ballen, Chalene Bezzina, Callum McDiarmid, Rasmus Johansson, Joanna Sudyka, Mathieu Giraudeau, Adele Haythornthwaite**) when it came to catching, caring for, and collaborating on researching the little darlings. A shout out to **Mel Elphick** for the wisdom of advising me to write these acknowledgements in advance.

I'm also grateful to the other herpers I've met along the way. The laid back attitudes of Australian herpetologists (particularly the ASH regulars), combined with their clear love of the animals they study, is a solid reminder that it's important to enjoy the process of research. The sheer level of excitement when I announced at ASH 2015 that I'd found a brown snake in a toilet is something that will stick with me for life.

I was clearly destined to be a massive nerd, but I have to thank **my parents** for really nurturing it when I was a kid. Between the sheer number of nature documentaries consumed on Saturday evenings, the many gifted and talented weekends, and the annual pass to the Kidseum in Merrylands they more than enabled my interest in science and how stuff works.

Thanks also to my bff/poorly trained racoon, **Harriet O'Sullivan**. The hours you spent at Kuleto's listening to me whine about anything and everything have not been in vain.

In a twist of fate, Mats' resignation from Usyd afforded me the chance to do some research in Gothenburg in November 2016. Keen to explore in the month I was there I agreed to a date with a local girl who promised to show me baby chameleons at the Universeum (a sort of zoo-Questacon hybrid). Despite our initial intentions this turned into a long distance relationship and **Tamm Sjödin** and I have been together ever since. Tamm, thank you so much for the support you've given me, being able to vent frustrations and celebrate

victories with you has made the journey that much better. Always keen to help out in any way you can, you've been my rock. Even if that rock was unfortunately sometimes over 16,000 km away.

My ultimate thanks goes to the dragons and snakes themselves. No one and nothing contributed more to the research or my understanding and appreciation of evolutionary biology than they. May climate change be averted such that Australian summers never become too hot, nor Canadian winters insufficiently cold.

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ABSTRACT

Telomeres, short tandem repeats of TTAGGG located at the ends of chromosomes, are dynamic structures which may be affected by the life history strategy of an organism. Telomeres shorten during cellular replication and may also be damaged by reactive oxygen species (ROS). Conversely, telomeres can be lengthened by telomerase activity and ROS may be countered by antioxidant production. Telomere length (TL) can affect the repair capacity of tissues and is implicated in the ageing process. Telomere dynamics vary among species, and also may vary among individuals or populations within species. Research comparing life histories and telomere dynamics has primarily focussed on interspecific comparisons which may be confounded by genetic variation. By studying the telomeres of polymorphic species with unusual life history strategies, genetic variation is minimised and we may be able to identify trends that could otherwise be obscured. Using quantitative PCR I investigated the telomere dynamics of two reptile species with unusual life history strategies, painted dragons *Ctenophorus pictus,* and red-sided garter snakes *Thamnophis sirtalis parietalis*.

Painted dragons live for approximately one year in the wild and males vary in their head colour (red, orange, yellow and blue) and presence or absence of a yellow gular bib. The morphs vary in life history strategy and behaviours with red-headed males dominating yellow-headed males in direct competition while yellow males outcompete red males in sperm competition trials. Males without bibs are more resistant to stress, but potentially less attractive to females than are bibbed males. Males patrol territories and seek mates while females are sedentary and cryptic. I investigated the TL of blood, brain, heart, liver, and spleen of male and female juvenile dragons and observed that female dragons have longer telomeres than do males. The largest observed difference was in brain telomeres

where male TLs were approximately half as long as those of females, potentially due to the rapid brain development required to effective patrol territories. I also measured blood TL of male dragons during and at the end of the mating season to investigate whether their rapid ageing was reflected in telomere dynamics. Yellow and bib-less males had longer telomeres than did the other morphs, suggesting that telomere length relates positively to self-maintenance. Finally, I investigated the relationship between morphs and sperm TL. Contrasting with the result for blood TL, orange males had the longest telomeres and bib-less males had shorter telomeres than those with bibs. The differences in TL in blood and sperm may relate to differences in reproductive expenditure.

Red-sided garter snakes hibernate for eight months of the year in communal dens and mating occurs upon emergence in large mating aggregations. These aggregations are heavily male biased, with males attempting to court females for up to three weeks and remaining aphagic throughout. By contrast, females prioritise resource acquisition and cellular maintenance. I investigated the difference in blood telomere dynamics with age between the sexes and observed that females had longer telomeres than did males. Female snakes also maintained TL as they aged. By contrast, males experienced an ontogenetic decline in TL, likely due to the trade-off between self-maintenance and their high reproductive investment. I also assessed the TL of sperm collected from the copulatory plugs of females that had recently mated. Sperm TL correlated with blood TL and males that were longer and grew relatively faster had greater sperm TL. This correlation may be driven by resource acquisition ability or due to variations in testosterone, which can act as an oxidant and constrains growth in this species.

The results of my studies indicate that telomere dynamics can vary greatly between and even within individuals through time. Research at an organismal level that considers life

history strategy relative to conspecifics may help illuminate trends that would be difficult to detect through interspecific comparisons. Polymorphic species provide a powerful model with which to test interactions between telomere dynamics and life history strategies.

CHAPTER 1: General Introduction

The life history strategy of an organism describes how resources are allocated to processes such as growth, reproduction and cellular maintenance (Stearns, 1976, Ricklefs, 2010). Resources are limited and natural selection typically results in a pattern of resource allocation that maximises reproductive success, with trade-off optima set by selection (Van Noordwijk & Dejong, 1986, Stearns, 1989). Allocation of resources to reproduction over cellular maintenance may result in an organism that "lives fast", i.e. an organism that produces many offspring quickly but ages rapidly (Promislow & Harvey, 1990), where ageing consists of cellular sencesence and reduced physiological functioning (Liu, 2014). Alternatively, selection may favour cellular maintenance, reducing reproductive output at any one time but allowing for more reproductive events. The particular life history strategy of an organism may be influenced by environmental factors such as reliability of resources and thermal variation (Ricklefs & Wikelski, 2002). The influence of these factors on somatic 'wear and tear' versus maintenance may be observed at the molecular level and my thesis will focus on the relationship between life history strategies and telomeres.

Telomeres are short tandem DNA repeats of TTAGGG located at the ends of chromosomes (Blackburn & Gall, 1978, Blackburn, 2005, Hug & Lingner, 2006) and may have several functions: they a) prevent the loss of coding DNA during cellular division, due to the "end replication problem" (Harley et al., 1990, Hug & Lingner, 2006), b) assist in aligning chromosomes correctly during metaphase (Dynek & Smith, 2004, Lin et al., 2004), c) prevent accidental chromosome fusion by to the repair mechanisms of the cell (Lee et al., 1998, Houben et al., 2008), and d) are easily damaged and can act as guides for triggering cellular senescence, thus preventing the replication of unstable cells (Kawanishi et al., 2001, von Zglinicki, 2002, von Zglinicki, 2003, Kawanishi & Oikawa, 2004).

Telomeric attrition often occurs as an organism ages due to chromosomal replication and is exacerbated by the damage caused by reactive molecules (e.g., nitric oxide or reactive oxygen species, ROS, Chen et al., 2007) and their by-products (Marnett, 1999). In the current thesis, I will focus on telomere attrition caused by reactive oxygen species, which are molecules and free radicals formed from molecular oxygen that may cause oxidative damage (Turrens, 2003). Macromolecules, such as DNA, are particularly susceptible to oxidative damage due to their relatively low turnover. Furthermore, telomeres are particularly vulnerable to oxidative damage as the triple G structure that forms part of their sequence is highly susceptible to oxidation (Kawanishi et al., 2001, Oikawa, 2001). Damage caused to telomeres by ROS may become permanent when chromosomal replication occurs if it is not repaired, causing the next generation of cells to have shorter telomeres (Kawanishi & Oikawa, 2004). However, attrition may be reversed through activity of the enzyme telomerase (Greider, 1990, Blackburn, 2005), or the telomere sequence may be repaired by several site-specific systems (e.g., BER, Olsson et al., 2018a and references therein). Telomerase contains an RNA template which can extend telomeres (Greider, 1990). Although telomerase activity varies greatly across taxa, it is often restricted to highly proliferative cells. Elongation of telomeres may prevent the triggering of senescence, leading to the replication of unstable cells and potentially result in cancer (Monaghan, 2010). Telomeric attrition may also be reduced through the activity of endogenous or exogenous antioxidants which counter and neutralise ROS (Magwere et al., 2006, Valko et al., 2007, Badas et al., 2015). The exact balance of cellular division, telomerase, ROS and antioxidants is influenced by the life history strategy of the organism.

Although relationships between telomere dynamics and life history strategies have been identified, they are not fixed but may change through life, and evolve over time. The rate of ageing may be more strongly related to telomeric attrition than to absolute length of

the telomeres (Haussmann et al., 2003, Bize et al., 2009). This makes intuitive sense as reductions in cellular maintenance processes, such as antioxidant production, typically cause faster ageing (Olsson et al., 2012). Shorter-lived organisms may have longer telomeres and often experience greater telomeric attrition than do other closely-related taxa (Francis et al., 2006). In some species, juveniles with longer telomeres live longer (Haussmann & Mauck, 2008, Heidinger et al., 2012). Heritability of telomere length has been documented in some species, but not others (Olsson et al., 2018b). Males often have shorter telomeres than females, but the opposite situation has also been observed (Barrett & Richardson, 2011). This variation among different species in telomere traits and dynamics challenges our ability to draw conclusions about the relationship between telomere dynamics, life history strategies, and evolutionary inference.

Substantial research has been conducted on the telomere lengths and sequences of the classic model organisms such as nematodes, fruit flies, zebrafish, African clawed frogs, chickens and mice (Gomes et al., 2010). The study of telomerase knock-out mice and zebrafish has demonstrated the importance of telomerase activity and telomere maintenance for longevity (Henriques et al., 2013, Lee et al., 1998). Telomerase deficiency has also been linked with increased carcinogenesis in mice (Rudolph et al., 1999). In *Drosophila* telomere extension is performed by retrotransposable elements, rather than telomerase resulting in a more irregular telomere structure (Pardue & DeBaryshe, 2008). Telomerase activity in chickens decreases throughout development in a similar manner to humans, except that their telomeres are approximately ten times longer (Delany et al., 2003). Research using model organisms typically focusses on the underlying molecular functioning of telomeres often with the intention of investigating human diseases, rather than the relationship between telomere dynamics and life history strategies. The usage of model organisms for investigating life history strategies and telomere dynamics presents

two main problems. Firstly, model organisms were often chosen as they were perceived to be useful for a particular topic of research (Hunter, 2008), although they are frequently considered to be representatives of particular taxa. Whether they are particularly representative or not, model organisms do not typically have unusual life history strategies where we may be able to identify differences in telomere dynamics. Secondly, research involving model organisms is typically laboratory based, and many of the traditional stresses experienced by free-living animals have been removed. An environment with abundant food and no predation is less likely to produce trade-offs and may obscure trends in telomere dynamics that would otherwise be apparent in a wild population.

As such, research involving wild populations may be preferable but have thus far been more limited. Wild populations are both more difficult and time consuming to access, making it harder to obtain sufficient sample sizes. Studies have frequently been more observational than manipulative due to the difficulty of applying such manipulations (e.g. restricting the diet of some individuals in a freely foraging population), although there have been some successful exceptions (Young et al., 2017). It is, therefore, possible to investigate the telomere dynamics of wild populations through careful experimental design. A study of five bird species reinforced the relationship between telomere stability and life span and may have identified a long lived bird that experiences telomere extension with age (Haussmann et al., 2003). In male sand lizards, the costly process of tail regrowth results in telomere attrition (Olsson et al., 2010). The relationship between parental age at conception and offspring telomere length has also been investigated in multiple non-model species (Froy et al., 2017, Olsson et al., 2011). Thus, research on the telomere dynamics of wild populations and non-model organisms has been performed successfully. By allowing ourselves to consider a much broader range of species we may be able to identify species

with particular life history strategies that are likely to reveal otherwise difficult to observe trends in telomere dynamics.

Furthermore, most of our understanding of telomere dynamics has come from interspecific comparisons, involving a combination of model and non-model organisms. These are necessarily limited in what they can reveal, as interspecific differences in genetics and environment will obscure underlying trends (Dugdale & Richardson, 2018). A more powerful approach may be to study species which exhibit intraspecific differences in life history strategy. Polymorphisms exist whereby a species contains individuals of more than one distinct form, a diversity that can be stable across evolutionary time (Ford, 1940, Richman, 2000). The most common example of polymorphism is sexual dimorphism (Lande, 1980, Shine, 1989). However, polymorphisms also exist with respect to colour and to molecular traits such as blood type (Wellenreuther et al., 2014). Telomere dynamics are often subtle, thus intraspecific research on polymorphic species with variable life history strategies may help clarify otherwise hidden trends.

Thus, I studied the telomeres of two wild polymorphic reptiles in an attempt to clarify trends in telomere dynamics and life history strategies. The painted dragon lizard, *Ctenophorus pictus*, is a short-lived (~1 year in the wild) Australian species with sexual dimorphism and colour polymorphic males (Olsson et al., 2007b, Olsson et al., 2008, Healey & Olsson, 2009). The red-sided garter snake, *Thamnophis sirtalis parietalis*, is a North American species with extreme sexual dimorphism with respect to reproductive effort and size (Gregory, 1977, Crews & Garstka, 1982).

Painted dragons are found in semi-arid parts of the southern half of Australia, and the population used for this research is located in Yathong Nature Reserve, NSW. This species

rapidly ages and most individuals only participate in a single mating season (Olsson et al., 2007b). Females are sedentary and cryptic while males actively maintain and patrol territories and are brightly coloured (Olsson et al., 2007b). Each male has blue sides, a distinct head colour (red, orange, yellow, or "blue" – a lack of head colour that makes their blue sides seem more predominant) and may have a yellow gular bib. Head colour morphs have varied in frequency since research on this population began, thus most life history research has focussed on red-headed and yellow-headed males and on the functional significance of bib colouration (Olsson et al., 2007b). Both red and yellow males maintain territories, but red males outcompete yellow males in physical contests and have higher testosterone concentrations (Healey et al., 2007, Olsson et al., 2007a). By contrast, yellow males mate more quickly and outcompete red males in sperm competition (Olsson et al., 2009). Males without bibs are more resistant to stress (Healey & Olsson, 2009), but males with bibs may be more attractive to females (McDiarmid et al., 2017). Thus, multiple life history strategies can be observed within this species, potentially making them an ideal model for investigating the subtleties of telomere dynamics.

Chapter 2 compares the similarity of telomere length between blood cells and several other tissue types in male and female juvenile dragons. Most previous research on telomere dynamics has focussed on blood cell telomere length, due to ease of collection and its non-destructive nature. It is rarely tested, but typically assumed, that the telomere dynamics of blood are reflective of the dynamics of other cell types. Here I test this assumption while also investigating whether the sexual dimorphism of the species alters telomere dynamics in a tissue-specific manner. Similarly, research on telomere dynamics often focuses on mature adults, where reproductive effort may affect the observed dynamics. In this chapter I show that differences in telomere dynamics between the sexes may arise prior to maturity.

Chapter 3 assesses differences in sperm telomere dynamics, velocity and number between the morphs. Sperm telomere length may determine the telomere length of offspring and potentially their lifespan. The reproductive success of offspring determines a male's fitness, therefore sperm telomere length may be an important trait that varies with life history strategy. Sperm velocity and number are examined, to determine whether sperm telomere length correlates with typical measures of sperm quality or whether a trade-off occurs. This chapter indicates the importance of considering gametic and somatic cells when investigating telomere dynamics.

Chapter 4 investigates the relationship between the various male morphs and blood telomere dynamics across the mating season. As the morphs have different life history strategies, this provides an effective way of investigating how telomere dynamics vary with life history strategy while minimising the genetic variation resulting from among-species comparisons. At present our ability to determine whether telomeres cause ageing, or are just a measure of it, is limited as there is currently no simple method to manipulate telomere length *in vivo*. Thus this chapter also contains an attempt at telomere manipulation through the administration of a purported telomerase activator.

Red-sided garter snakes are viviparous colubrid snakes native to North America (Gregory, 1974). The study population I have used in my research is located in the Interlake Region of Manitoba, Canada. These snakes have evolved an extreme life history strategy to cope with the environmental conditions. As temperatures drop below freezing for a large portion of the year the snakes overwinter communally for approximately eight months in limestone fissures which may contain tens of thousands of snakes (Aleksiuk & Stewart, 1971, Crews & Garstka, 1982, Krohmer et al., 1987). When spring arrives the snakes emerge *en masse* and form large mating balls (Aleksiuk & Gregory, 1974). These mating balls are highly

male-biased as males remain at the den for approximately three weeks to attempt to mate with females, while females migrate to feeding grounds once sufficiently warm (Shine et al., 2001). Females are larger than males and, despite almost always being mated with upon emergence, typically only produce young every two years (Crews et al., 1985, Shine et al., 2003, Shine & Mason, 2005, Gregory, 2006, Gregory, 2009). By contrast, males produce sperm prior to hibernation, and are aphagic during the mating season (Aleksiuk & Stewart, 1971, Crews & Garstka, 1982, O'Donnell et al., 2004). Males also produce energetically costly mating plugs which contain sperm and make it difficult for other males to copulate with an already-mated female (Shine et al., 2000, Friesen et al., 2013, Friesen et al., 2015). Thus selection favours the allocation of resources to reproduction in males and to growth and maintenance in females, resulting in females typically living several years longer. The extreme difference between the sexes, partially induced by the environmental conditions, makes them ideal candidates for investigating variations in telomere dynamics with life history strategies.

Chapter 5 compares ontogenetic differences in telomere dynamics between male and female garter snakes. Determining how telomere length varies with age is important as the rate of telomere change may more effectively predict lifespan than can actual telomere length. Due to the difference in lifespan and reproductive strategies between the sexes we would predict variation in telomere dynamics. Although my data are cross-sectional, a difference in telomere dynamics between the sexes supports the hypothesis that average lifespan can be predicted by changes in telomere length.

Chapter 6 investigates the relationship between sperm telomere length and growth rates. As males have a short period to gather resources which are used for both growth and reproduction, this work investigates whether resource abundance and the time period where cellular division occurs can affect telomere dynamics. This chapter also compares

results with those in Chapter 5, investigating how somatic and gametic telomere dynamics vary in males.

In order to measure telomere dynamics I used quantitative polymerase chain reactions (qPCR). In qPCR, telomere lengths are amplified, using specially designed primers, along with a non-variable reference gene and the ratio of fluorescence between the telomere signal and reference gene signal gives a relative measure of the total amount of telomere content in a sample (Cawthon, 2002). Several other methods of telomere analysis have also been developed such as telomere restriction fragment analysis (TRF), quantitative fluorescence *in situ* hybridisation (qFISH), flow fluorescence *in situ* hybridisation (flow FISH), single telomere length analysis (STELA) and telomere shortest length assay (TESLA, Lai et al., 2018). I chose qPCR for a few reasons. The methodology has existed for some time and minimum standards for publishing, to ensure robust results, have been established (Bustin et al., 2009, Cawthon, 2002, Criscuolo et al., 2009). qPCR has been used in many life history studies (e.g. Botha et al., 2012, Froy et al., 2017, Hatase et al., 2008, Rollings et al., 2014, Voillemot et al., 2012), compared with processes such as STELA and TESLA which are designed largely for a clinical setting (Lai et al., 2018). The method has a high throughput compared with the other methods, with the exception of flow FISH. qPCR may also be performed with tissue samples that have been frozen, unlike flow FISH and qFISH. qPCR is also cheaper than flow cytometry. However, qPCR does have a few disadvantages. Primarily, qPCR is only able to determine relative telomere length compared with other samples involved in a study, limiting the comparisons that may be made between studies and species (Cawthon, 2002). The methodology also only determines average telomere length, while telomere length may vary between chromosomes potentially affecting the life history of an organism (Cawthon, 2002, Lai et

al., 2018). The primers used are also unable to distinguish between terminal telomeres, which are the focus of my research, and interstitial telomeres that may be found throughout chromosomes (Foote et al., 2013). As such, the usage of qPCR requires careful consideration of the limitations in order to design experiments that can obtain biologically relevant results.

My research attempts to clarify the covariation between telomere dynamics and life history strategies. Much research on telomere dynamics has focussed on traditional model organisms without consideration of whether they are the ideal species for investigating life history. Thus my research has two main aims. Firstly, I investigate how telomere dynamics vary between and within individuals, and determine possible explanations for the observed trends by examining them from the perspective of life history and telomere dynamics and evolution. Secondly, I attempt to demonstrate the importance of considering life history strategy when determining ideal species for investigating telomere dynamics. The chapters of this thesis are presented as either published papers or submitted drafts. As such, some repetition of content is unavoidable.

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CHAPTER 2: Sex- and tissue-specific differences in telomere length in a reptile

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Abstract

The usage of telomere length (TL) in blood as a proxy for the TL of other tissues relies on the assumption that telomere dynamics across all tissues are similar. However, telomere attrition can be caused by reactive oxygen species (ROS) which may vary with metabolic rate, which itself varies across organs depending upon the life history strategy of an organism. Thus we chose to measure the telomeres of various cell types in juvenile painted dragon lizards, *Ctenophorus pictus*, given their unusual life history strategy. Individuals typically only experience a single mating season. We measured the TL of male and female dragons using qPCR and observed that TL varied with tissue type and sex. Telomeres of blood cells were longer than those of liver, heart, brain, and spleen, and females had longer telomeres than males. Brain telomeres in males were approximately half the length of those in females. Telomeric attrition in the male brain may be due to the need for rapid learning of reproductive tactics (territory patrol and defence, mate-finding). Significant correlations between the TL of tissue types suggest that blood TL may be a useful proxy for the TL of other tissues. Our comparison of organ-specific telomere dynamics, the first in a reptile, suggests that the usage of blood TL as a proxy requires careful consideration of the life history strategy of the organism.

Keywords: *Ctenophorus pictus*, life history, painted dragon lizard, reptiles, sex differences, telomeres

Introduction

Telomeres, short tandem repeats of TTAGGG located at the ends of chromosomes (Blackburn, 2000; Blackburn & Gall, 1978), are highly dynamic structures that are tightly linked to the life history strategies of species (Gomes, Shay, & Wright, 2010; Haussmann & Marchetto, 2010; Rollings, Uhrig, et al., 2017), and even morphs within species (Rollings, Friesen, et al., 2017). Telomeres are highly dynamic because they are affected by several factors, to a degree which may be determined by the life history strategy of the organism through the allocation of resources (Haussmann & Marchetto, 2010). Every round of cellular division typically shortens telomeres, due to incomplete chromosomal replication (Olovnikov, 1973), but telomeres may be extended again through activity of the enzyme telomerase (Giardini, Segatto, Silva, Nunes, & Cano, 2014; Greider, 1990).

Telomeres may also become shorter when damaged by reactive oxygen species (ROS, Houben, Moonen, Schooten, & Hageman, 2008, Olsson, Friesen, et al., 2018, Selman, Blount, Nussey, & Speakman, 2012, von Zglinicki, 2002). Reactive oxygen species are produced in mitochondria when electrons leak from the electron transport chain during oxidative phosphorylation and interact with molecular oxygen (Beckman & Ames, 1998; Turrens, 2003). As oxidative phosphorylation produces most of an organism's ATP (Bertram, Gram Pedersen, M., Luciani, D. S., & Sherman, A., 2006), an increase in metabolism may increase ROS production and cause oxidative stress. However, ROS may be countered by antioxidants (Magwere et al., 2006; Monaghan, Metcalfe, & Torres, 2009), reducing telomeric attrition. Organisms that prioritise reproduction or growth over cellular maintenance (such as antioxidant production) often age more quickly, have shorter life spans, and experience higher rates of telomere loss (Promislow & Harvey, 1990; Ricklefs & Wikelski, 2002). Metabolic rates may vary between organs and tissues (and are frequently correlated with organ size, Piersma, Gudmundsson, & Lilliendahl, 1999, Wang

et al., 2010), often influenced by life history strategy (Ricklefs & Wikelski, 2002). Thus, ROS concentrations may also vary among tissues, and tissues or organs may experience unique telomere dynamics, depending upon the life history strategy of the organism. We would predict that organs with higher oxidative stress would experience higher rates of telomeric attrition.

Many studies of telomeres, particularly those in evolutionary biology, focus on telomere lengths (TL) in whole blood or white blood cells (Badas et al., 2015; Barrett & Richardson, 2011; Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Froy et al., 2017; Lopez-Arrabe et al., 2018; Olsson, Wapstra, & Friesen, 2018; Rollings, Uhrig, et al., 2017). This focus on blood is largely due to ease of collection, relatively low invasiveness, and because it allows repeated collection of samples. However, without knowing whether blood TL are representative of the various organs and tissues within an organism, it is difficult to determine whether blood TL provides meaningful inferences about the organism as a whole.

Research on zebra finches has shown robust correlations between telomere dynamics in red blood cells and the brain, liver, and spleen (Reichert, Criscuolo, Verinaud, Zahn, & Massemin, 2013). Telomere lengths correlate between tissues in humans, dogs, and pigs (Benetos et al., 2011; Daniali et al., 2013; Fradiani, Ascenzioni, Lavitrano, & Donini, 2004; Friedrich et al., 2000), although a study of human cadavers testing a wide range of tissues found few such correlations (Dlouha, Maluskova, Lesna, Lanska, & Hubacek, 2014). Telomere lengths are also consistent between tissues in zebrafish (Lund, Glass, Tolar, & Blazar, 2009), whereas a study on Wistar rats reported shorter telomeres in males than females in kidneys, liver, pancreas, and lungs, but not brain (Cherif, Tarry, Ozanne, & Hales, 2003). This study also reported telomeric attrition with age in all tissues for both sexes except for the brain. Thus, while correlations between cell types have been

observed, research has focussed on a limited range of taxa. In order to determine whether trends of telomere correlation are consistent across a broader range of taxa, in particular those with unusual life history strategies, we designed this study on a short-lived Australian lizard.

We assessed TL across a range of tissues in juvenile painted dragon lizards, *Ctenophorus pictus*, a reptile species with a notable life history strategy (Figure 1). These dragons are short-lived (~1 year in the wild, Olsson, Healey, Wapstra, et al., 2007) agamid lizards found in semiarid regions in the southern half of Australia. Males are polymorphic in color (red, orange, yellow, and a lack of head color referred to as "blue," Healey, Uller, & Olsson, 2007, Olsson, Healey, & Astheimer, 2007, Olsson, Schwartz, Uller, & Healey, 2009, Rollings, Friesen, et al., 2017) and exhibit morphspecific adult behaviors as they patrol territories and court females. By contrast, females are monomorphic, sedentary, and cryptic (Olsson, Healey, Wapstra, et al., 2007). Territories of individual males may be up to 50 m in length and are patrolled to prevent ingress by neighboring or invading males (Healey et al., 2007). Most dragons only participate in a single mating season, thus males must rapidly learn the behaviors of neighboring males and understand the structures of their territories to increase their chances of successful reproduction. Therefore, their ability to process such information is likely under strong selection. The importance of effective patrolling may be reflected in brain structure, with a comparatively larger optic tectum in the painted dragon than in other closely related species (Hoops, Vidal-García, et al., 2017). The tectum, the primary processing center for visual information (Bischof & Watanabe, 1997), is involved in processing agonistic visual displays perceived in signaling (McDonald, Paul, & Hawryshyn, 2004), and thus may be important for observation and patrol of a territory. Male painted dragons also have a larger medial preoptic nucleus (MPON) than do conspecific females (Hoops, Ullmann, et al., 2017). The MPON

facilitates male reproductive behavior, such as mate-seeking and aggression (Balthazart & Ball, 2007; O'Connell & Hofmann, 2011). The sex difference in MPON size suggests that brain function is important in male dragons for successful reproduction. Territorial behaviors among males begin before reaching maturity (Healey et al., 2007), with morph type influencing the probability of winning contests, suggesting that the brain develops before other reproductive structures. As females do not patrol or maintain territories, it is likely that they do not need to devote resources to rapid brain development. This species is less sexually dimorphic in body size than are most other *Ctenophorus* species (Hoops, Ullmann, et al., 2017), and the sex of juvenile dragons is indistinguishable (without inspection for hemipenes) until males develop colors as they approach maturity. The lack of size dimorphism suggests that males and females invest similarly in growth rates, and thus the cost of rapid brain development in the males is likely paid elsewhere. Males may prioritise growth and brain development over cellular maintenance, possibly downregulating antioxidant production and causing higher oxidative stress, which may increase telomere attrition. We, therefore, predict that juvenile male dragons will have shorter telomeres than females, especially in the brain (as it is a site of rapid development). By extension we predict there will be correlations in TL between the tissue types, with potentially the weakest correlation between brain and the other tissues due to the life history strategy of the males described above. Our comparative study of tissue- and sex-specific telomere dynamics, the first in a reptile, investigates telomeres while also considering the internal and external environment of the species.

Figure 1: Plate of examples of *Ctenophorus pictus*. a) Juvenile dragons. b) Adult male. c) Adult female (the abdominal skin flaps on the female indicate recent oviposition).

Methods

Study organism

Mature (~9 months old) female dragons (*Ctenophorus pictus*, W. Peters, 1866) were caught by noose or hand at Yathong Nature Reserve, NSW, Australia (145°35′E; 32°35′S) and taken to holding facilities at the University of Sydney in October 2015 where they were housed for the duration of the experiments. Animals were collected under a permit issued by NSW National Parks and Wildlife Service (SL100352), and experiments were conducted in accordance with University of Sydney ethics approval (AEC-2013/6050). Females were housed in pairs in opaque plastic tubs (330 × 520×360 mm) with sandy

substrate and exposed to a 12 hr light: 12 hr dark cycle. The lizards were fed mealworms and crickets, dusted with calcium and multivitamins, to satiation every day, and the cages were misted with water once a day. Heat lamps and ceramic hides were provided to allow the lizards to thermoregulate to their preferred body temperature (36°C; M.O., unpublished data obtained from cloacal temperature readings in the wild). A mound of moist sand was available in each tub to allow females to burrow and lay eggs. Females were checked for oviposition each day, as apparent from skin flaps around the abdomen. Overall, 18 females produced 22 clutches and 89 eggs in total between 4 November 2015 and 6 January 2016. The eggs were removed and weighed to the nearest 0.1 g. As part of an investigation on the effects of temperature on TL, the individual eggs from each clutch were then placed sequentially in one of four incubators (27, 30, 32, 36°C, ±0.5°C) to separate clutch and temperature effects. Eggs were half-buried in a 1:7 mix of water to vermiculite in sealed, transparent, containers in each incubator. Containers were sealed to reduce evaporation of water but aerated weekly. Eggs were checked daily for nonviable eggs, which were removed. Seventy-three dragons hatched and 16 nonviable eggs were removed (most from the 36°C incubator, which is warmer than estimated incubation temperature in the wild: approximately 30°C, M.O., unpublished data). Hatchlings were sexed (determined by the presence or absence of hemipenes), weighed to the nearest 0.1 g, and snout–vent length (SVL) and total length were measured to the nearest mm. Each individual was marked with a unique toe-clip pattern for identification. Individuals were then placed in groups in tubs with the same conditions as the adult females, with the exception of the sand mound. Hatchling dragons were fed pinhead crickets, dusted in calcium and multivitamins, to satiation every day. Mortality is high in juvenile painted dragons but tubs were checked multiple times a day and any dead individuals were promptly removed.

Sample collection

In March 2016, the 24 remaining juveniles (14 female, 10 male, ranging in age from 97 to 140 days, 1 female and 3 males incubated at 27°C, 6 females and 5 males incubated at 30°C, 5 females and 2 males incubated at 32°C, 2 females incubated at 36°C) were euthanized by decapitation. Prior to decapitation individuals were first sedated by cooling in a refrigerator for 10 min and in a freezer for 2 min. They were sexed again to confirm prior assignations, weighed to the nearest 0.1 g, and SVL and total length measured to the nearest mm. Whole blood, whole brain, heart, liver, and spleen were then collected. Each tissue type was placed in 300 µl of RNAlater (Sigma-Aldrich, Castle Hill, NSW, Australia) and stored at −80°C until DNA extraction.

Quantifying relative telomere length

To analyse relative telomere length (RTL; relative to the 18S gene), we first purified DNA from the collected tissues. The brain, heart, and liver were sliced into small pieces, and 50 µl of the diluted blood was used in the extraction. A DNeasy Blood and Tissue Kit (Qiagen, Chadstone, VIC, Australia) was used for extractions, according to the manufacturer's instructions. The protein kinase digestion step was run for 10 min for the blood, but the brain, heart, liver, and spleen required overnight incubation for complete digestion. RNase A (Qiagen, Chadstone, VIC, Australia) was added at the recommended concentration. The DNA concentration (ng/µl) and A260:A280 ratio of each sample were measured in duplicate using a Nanodrop (Thermo Fisher Scientific) and aliquots diluted to 10 ng/µl using the AE buffer provided in the DNA extraction kit. Only samples with a A260:A280 ratio between 1.7 and 1.9 and a concentration above 10 ng/µl were considered high enough quality to be used in analyses. Ultimately, 22 blood (12 female, 10 male), 18 liver (9 female, 9 male), 17 heart (8 female, 9 male), 16 brain (11 female, 5 male), and 10

spleen (6 female, 4 male) samples were of sufficient quality for use in the study. DNA was stored at −30°C.

Telomere length was measured using real-time quantitative PCR (qPCR) using SensiMix SYBR No-ROX Kit (Bioline, Sydney, NSW, Australia) and a Rotor-gene 6000 thermocycler (Qiagen, Chadstone, VIC, Australia) according to published protocols (Rollings, Friesen, et al., 2017; Rollings, Uhrig, et al., 2017) using techniques developed by Criscuolo et al. (2009) and Plot, Criscuolo, Zahn, and Georges (2012) with the 18S ribosomal RNA (18S) gene as the nonvariable copy number reference gene. The telomere primers used were Telb1 (5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′) and Telb2 (5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTAC CCT-3′, Cawthon, 2002). The 18S gene (92 bp amplicon in *Anolis*) was selected as the reference gene as it had previously been validated in reptiles (Plot et al., 2012; Rollings, Uhrig, et al., 2017). The primer sequences used were 18S-*F* (5′-GAGGTGAAATTCTTGGACCGG-3′) and 18S-R (5′- CGAACCTCCGACTTTCGTTCT-3′). Reactions were run in triplicate for each sample, with each run containing either Telb or 18S primers. Amplifications were carried out in a Rotor-Gene 6000 thermocycler (Qiagen, Australia) using an initial Taq activation step at 95°C for 10 min and a total of 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Each reaction had a final volume of 20 μl with 10 ng of DNA, forward and reverse primers used at a concentration of 250 nM, and $MgCl_2$ added for a concentration of 1.7 mM. 11.25 μl of the SensiMix SYBR No-ROX Master Mix was added per reaction. A melt curve was generated after each run over the temperature range of 60 to 95°C to ensure that there was no nonspecific product amplification (see appendix Figure S1 and Figure S2 for examples). All of the DNA samples for a given individual were included in the same run. No-template control reactions were run in triplicate for each primer set during every

qPCR run to ensure that there was no contamination. Standard curves were produced, using the pooled DNA from three randomly selected lizards, for both telomeres and 18S using fourfold serial dilutions to ensure consistent rates of amplification over a wide range of concentrations (60 ng/µl down to 0.05859 ng/µl with 6 different concentrations in total: Appendix Figure S3 and Figure S4) giving a linear dynamic range of 0.05859 to 60 ng/µl. The reaction was considered consistent when the linear correlation coefficient exceeded 0.985. The efficiency of the telomere amplification was 1.17 and the efficiency of the 18S amplification was 0.97, and samples all fell within the same concentration range as our standards. All runs included the same "golden standard" and also a no-template control to detect contamination. LinRegPCR 2016.0 (Heart Failure Research Centre, Ruijter et al., 2009, Tuomi, Voorbraak, Jones, & Ruijter, 2010) was used to analyse the qPCR data. The starting concentrations of telomere (T) and control gene (S; 18S) as determined with LinRegPCR were used to determine the relative telomere length with the calculation T/S. Telomeres and the control gene were assessed in separate runs. The mean interassay coefficient of variation for qPCR runs for telomere (n = 4) and 18S (n = 4) amplification were 2.98% and 0.59%, respectively, calculated using the golden standard. The intra-assay coefficient of variation for telomere and 18S runs were 1.09% and 0.90%, respectively. A general linear model found no significant difference in the distribution of the sexes among the plates ($F_{1,23}$ = 1.211, p = 0.283). To investigate possible run effects, we took the mean value for each triplicate as produced by LinRegPCR for the blood data to simplify the analysis. An ANOVA of the ln-transformed (for normality) 18S data showed no significant difference among the runs ($F_{3,18}$ = 0.795, p = 0.513) and neither did the runs containing telb ($F_{3,18}$ = 0.888, p < 0.466).

Statistical analyses

Analyses were conducted with SAS 9.4 (SAS Institute, Cary) and SPSS 25.0 (IBM, Armonk). RTL was ln-transformed in order to conform to normality, as verified with a Shapiro–Wilk test. Potential effects of body condition (BCI) were assessed by generating the residuals of a regression analysis of mass versus SVL at death. First, to test whether incubation at different temperatures had affected the results, a mixed model analysis of the relationship between RTL and incubation temperature, with individual ID and maternal ID as random factors, was conducted and found not significant ($F_{1,15.1} = 0.17$, $p = 0.6818$, $n = 78$). To further investigate potential effects of incubation temperature, Pearson's correlation coefficients of temperature, incubation time, and hatching mass were calculated. Pearson's correlations were conducted between RTL and SVL at hatching and death, mass at hatching and death, residual BCI at death, age at death, and growth rate (calculated as the difference between hatching and death SVL, divided by age). Pearson's correlation coefficients were also calculated for the telomeres of all combinations of the tissue types to test for similarity in telomere dynamics across the tissues. We chose not to apply a correction (e.g., Bonferroni) to this analysis despite the number of correlations tested for as the low sample sizes available limit our statistical power and increase the probability of type II errors. Application of a correction would only further increase the chance of type II errors and reduce our probability of detecting real effects (Nakagawa, 2004). To test for sex- and organ-specific telomere effects a mixed model analysis of the relationship between RTL and sex, organ type and a sex×organ type interaction was tested with ID included to control for multiple measures from the same individual. The sex×organ type interaction was not significant ($F_{4,73} = 1.206$, $p = 0.316$) but was retained in the final model as it resulted in a smaller −2 Res log likelihood. As organ type effects were detected, pairwise contrasts between each organ type were conducted. Sequential Bonferroni

adjustments were performed for all pairwise contrasts. As sex-based differences in RTL were found, GLMs testing sex-based differences in SVL at hatching and death, mass at hatching and death, residual BCI at death, age, and growth rate were performed to determine whether size differences might account for the variation in RTL.

Results

Effects of incubation temperature

Incubation temperature negatively correlated with incubation time (r = -0.8039, n = 24, p < 0.001) and hatchling mass (r = -0.5519, n = 24, p = 0.0052) but not hatchling SVL (r = -0.3587, n = 24, p = 0.0852).

Comparisons among organ types

The mixed model analysis ($F_{9,73}$ = 4.632, p < 0.001) revealed significant differences in RTL among organ types ($F_{4,73}$ = 6.964, p = 0.003). The dragons had a mean (±*SEM*.) blood RTL of 95.41 (±10.89), a mean heart RTL of 50.65 (±7.641), a mean liver RTL of 63.77 (±6.079), a mean brain RTL of 47.90 (±7.857), and a mean spleen RTL of 43.69 (±12.80). Blood RTL was significantly greater than heart RTL (t_{73} = 3.406, p = 0.01), brain (t_{73} = 4.772, p < 0.001), and spleen RTL (t_{73} = 3.399, p = 0.01). Liver RTL was significantly greater than brain RTL (t_{73} = 2.950, p = 0.03). Several significant correlations were found between the organ types (see Table 1 and Figure 2).

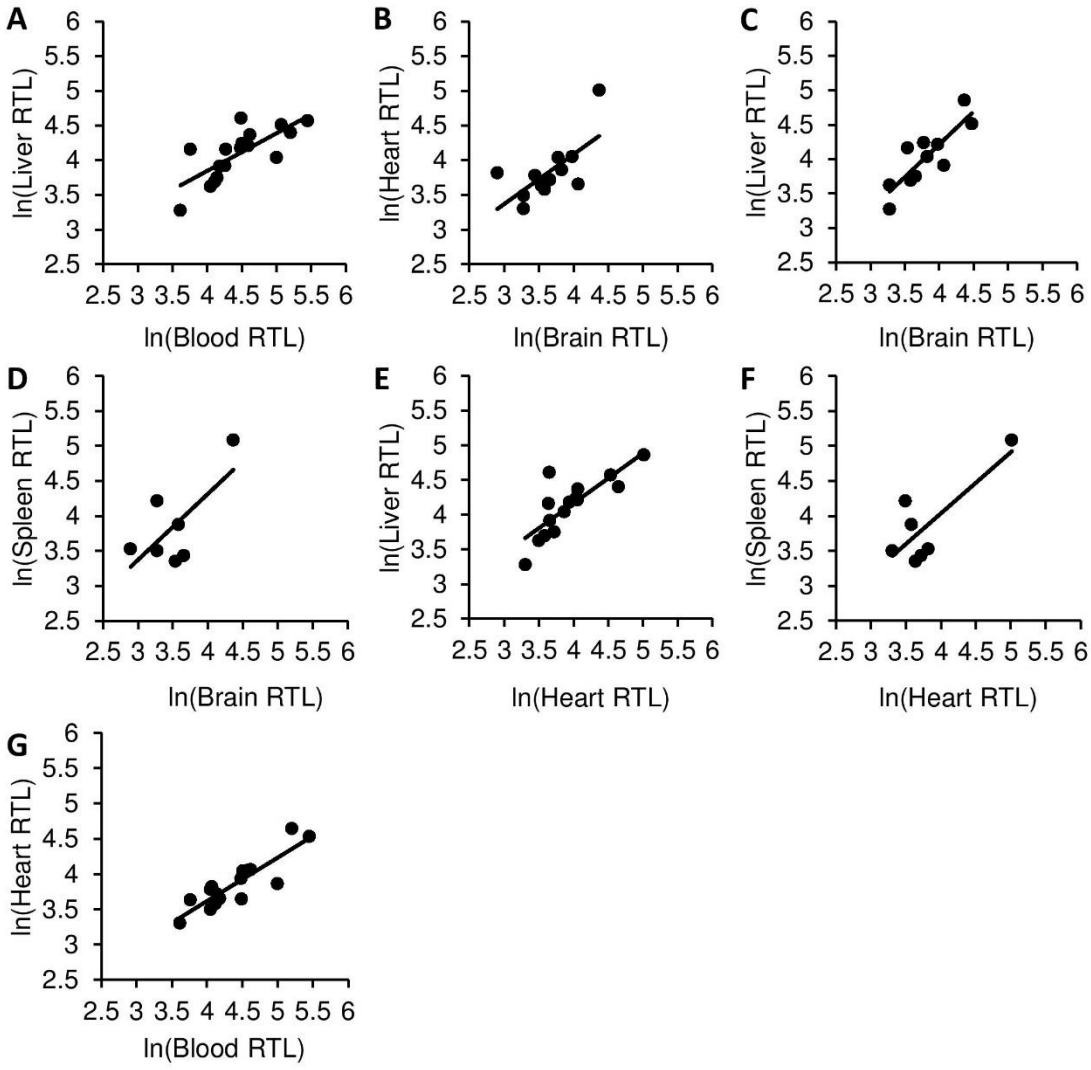

Figure 2: Significant Pearson's correlations between the ln-transformed relative telomere lengths (RTL) of the various tissue types collected from each individual. A) Correlation between liver and blood RTL (r = 0.74517, p = 0.0006, n = 17). B) Correlation between heart and brain RTL (r = 0.66747, p = 0.0177, n = 12). C) Correlation between liver and brain RTL (r = 0.84518, p = 0.0011, n = 11). D) Correlation between spleen and brain RTL (r = 0.68669, p = 0.0410, n = 9). E) Correlation between liver and heart RTL (r = 0.80095, p = 0.0003, n = 15). F) Correlation between spleen and heart RTL (r = 0.73080, p = 0.0253, n = 9). G) Correlation between heart and blood RTL (r = 0.87707, p < 0.0001, n = 16).

Table 1: Pearson's correlation coefficients for the relative telomere lengths of the different cell types. Calculations were performed on ln-transformed data. Significant correlations ($p < 0.05$) are bolded.

		Blood	Brain	Heart	Liver	Spleen
Blood	*r* *p* *n*	1.00000 22	0.38539 0.1736 14	0.87707 **<.0001** 16	0.74517 **0.0006** 17	0.59559 0.0906 9
Brain	*r* *p* *n*		1.00000 16	0.66747 **0.0177** 12	0.84518 **0.0011** 11	0.68669 **0.0410** 9
Heart	*r* *p* *n*			1.00000 17	0.80095 **0.0003** 15	0.73080 **0.0253** 9
Liver	*r* *p* *n*				1.00000 18	0.68344 0.0617 8
Spleen	*r* *p* *n*					1.00000 10

Sex-based effects on telomere length

Mixed model analyses revealed a significant difference in RTL between the sexes ($F_{1,73}$ = 9.268, p = 0.003, Figure 3), with females having longer telomeres. Female brain telomeres were approximately twice that of males (t_{73} = 3.227, p = 0.002) with a RTL approximately twice that of males. No significant body-size differences at either hatching or death were detected between males and females.

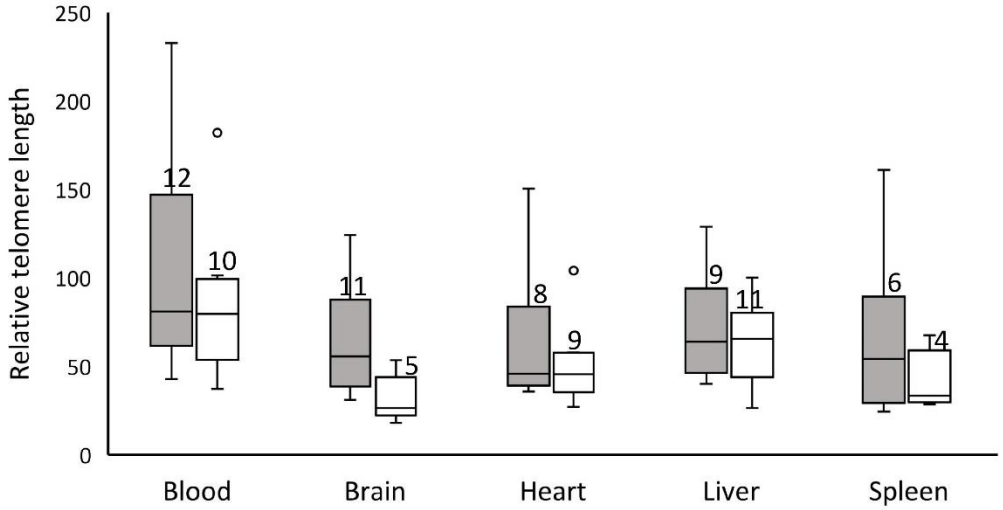

Figure 3: Comparison of the median and interquartile range (IQR, whiskers are minimum and maximum values, outliers are more than 1.5 times the IQR away from the third quartile) of female (grey) and male (white) relative telomere lengths (RTL). Ultimately, 22 blood, 20 liver, 17 heart, 16 brain, and 10 spleen samples from 24 dragons were used in the study. Mixed model analyses showed females had longer telomeres overall ($F_{1,73}$ = 9.268, p = 0.003) and *t*-tests showed female brain telomeres were longer than male brain telomeres (t_{73} = 3.227, p = 0.002, with Bonferroni adjustment applied). Statistical calculations were performed on ln-transformed data.

Discussion

We observed an overall consistency in telomere length (TL) among the organs of juvenile dragons, with females having slightly longer telomeres than males. However, our most striking result is that male lizards had very short telomeres in their brain cells. As these measurements represent a single time point, it is unclear whether males experience rapid telomere attrition after, or even prior to, hatching, or whether females maintain telomere length through telomerase activity. We did not measure the mass of the brain, but doubt that sex-specific differences in growth rate of the brain would be high enough to explain this discrepancy in TLs (given the low size dimorphism of the species, and the lack of difference in mass or SVL between the sexes in the present study). A more likely explanation, given that males must learn quickly in order to effectively patrol their territories, is that rapid brain development increases metabolic rate and thus ROS production. Investigations of tissue- and organ-specific ROS and antioxidant production could test this hypothesis. Regardless, this result indicates that telomere dynamics can vary greatly between tissues depending upon life history strategy.

We unfortunately do not know the morphs of the male dragons as they had not begun to produce their head colors. This may mean that our dataset was biased if males of a particular morph were overrepresented. Given the differences in behavior and reproductive tactics between red and yellow male dragons, we might predict differences in their brain telomeres also. Red dragons are aggressive, have high testosterone, and outcompete yellow dragons in combat bouts (Healey et al., 2007; Olsson, Healey, & Astheimer, 2007), whereas yellow dragons outcompete red dragons in sperm competition (Olsson et al., 2009). The aggression in red males may be facilitated by rapid MPON development, suggesting that red males may have shorter brain telomeres than yellow males. We have also previously found that red males have shorter blood telomeres than

yellow males (Rollings, Friesen, et al., 2017). While in the present study we have only found an indirect correlation of blood and brain telomere length (i.e., blood and brain TL both correlate with heart and liver TL), it is possible that red males may have shorter brain telomeres than yellow males. Given the different life history strategies and reproductive tactics contained within a single species, further research into the males of this species may reveal trends in morph-specific life history strategies and organ-specific telomere dynamics.

As females had longer telomeres than males, our results match several previous studies comparing TL between the sexes (Barrett & Richardson, 2011; Rollings, Uhrig, et al., 2017). However, most of these studies were performed on adult organisms, where the tradeoff of resource allocation toward reproduction may have initiated telomeric attrition (although see Schmidt et al., 2016 for tissue comparisons of gull embryos and juveniles). Our results show that the reproductive tactics of the male dragons may present costs prior to maturation. To clarify the generality of this phenomenon, future research needs to focus on a broader range of life stages in organisms to measure the costs associated with particular life history strategies across the entire life span.

Our results suggest that measurements of TL in the blood may serve as a useful proxy for TL in other tissues in dragons. Blood TL positively correlated with heart and liver TL and approached significance with spleen TL, despite the small sample size ($n = 9$). It is unclear why TLs in blood and brain of the same animal were not significantly correlated with each other. The lack of correlation is unlikely to be explained by the unusual telomere dynamics in the brain, as brain TL correlated with heart, liver, and spleen. Unfortunately, low sample size prevents testing for sex-specific differences in the correlation between blood and brain TL.

Telomeres in the blood were significantly longer than those in the brain, heart, and spleen. This contrasts with other studies which have reported either no difference or shorter blood telomeres compared with other tissues (Benetos et al., 2011; Daniali et al., 2013; Reichert et al., 2013). Little is known about the blood telomeres of reptiles which may simply be longer than the telomeres of other cell types, tissue-specific telomere lengths in reptiles require further investigation. Alternatively, the blood telomere length may be influenced by the methodology that we used. The much longer incubation used to fully lyse the cells in the organ samples may have caused more damage to the telomeres than to the reference gene due to their location on the chromosome, resulting in shorter telomere measurements. Further investigation is required to determine whether the methodology has influenced the results.

The correlations between tissues must be treated with some caution, as qPCR is unable to exclude interstitial telomeres, which we would predict to be consistent in length across tissues, and may artificially strengthen the relationships (Foote, Vleck, & Vleck, 2013). However, the lack of correlation between blood and brain TL, potentially caused by the difference in brain TL between the sexes, suggests that the observed significant correlations are caused by actual terminal telomeres. As we expect interstitial telomeres to remain consistent between cells, if interstitial telomeres were causing the correlations between tissues we should expect a significant correlation between blood and brain TL, despite potential sex-based differences. The lack of correlation between blood and brain telomeres gives us confidence that these correlations accurately indicate trends between the tissue types.

Our results provide some support for usage of blood TL as a proxy for the TL of other, harder to gather, and destructively sampled, tissues. However, correlations may not occur under all circumstances. We observed that telomere dynamics may vary between

tissues and sexes and may potentially be driven by the life history strategy of the species. While it may not always be possible or practical to compare blood and organ telomeres in a species, care should be taken to consider whether blood telomeres are likely to have the same dynamics as the telomeres of organs of interest. Careful consideration of life history strategies is vital for broadening our understanding of the mechanisms underlying telomere dynamics.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author contributions

Conceptualization: N.R., C.R.F, M.O.; Methodology: N.R., C.R.F, C.M.W, Investigation: N.R., C.R.F, R.J.; Data analysis: N.R., C.R.F., R.S., M.O.; Writing—original draft: N.R.; Writing—review & editing: N.R., C.R.F, C.M.W., R.J., R.S., M.O.; Supervision: C.R.F, C.M.W., R.S., M.O.

Data availability

Data is archived in Dryad (https://doi.org/10.5061/dryad.k2t648q).

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Appendix

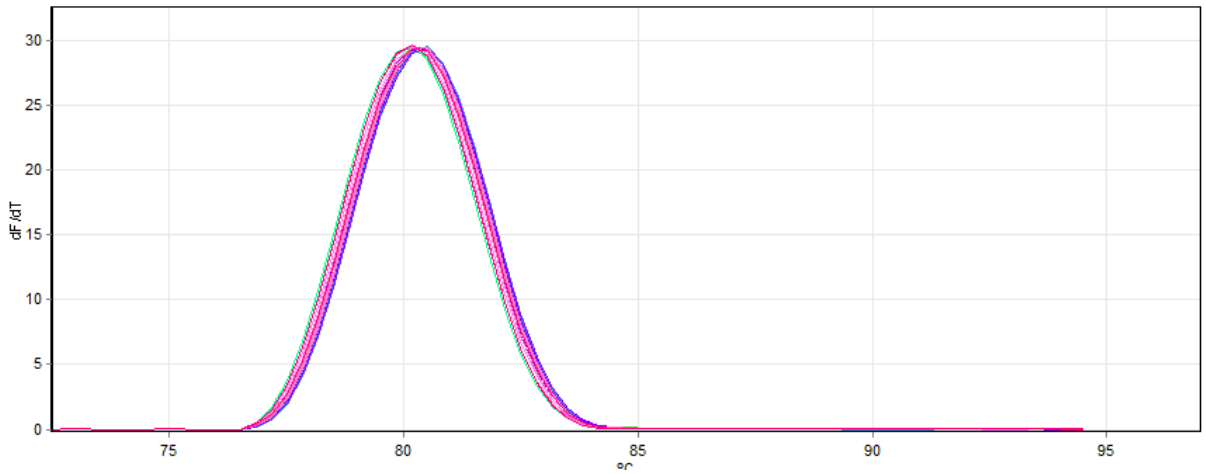


Figure A1: 18S melt curve, showing the amplification of a single product

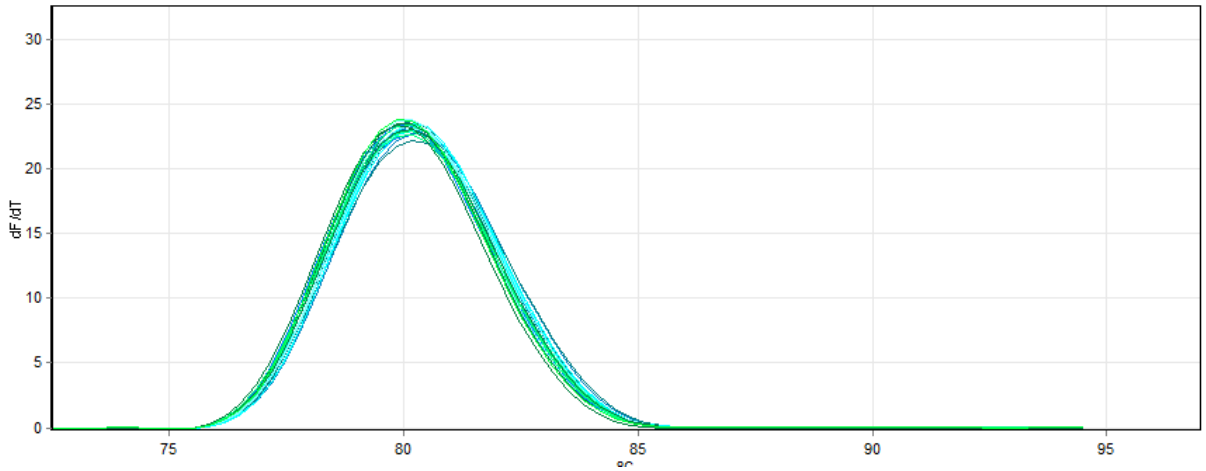


Figure A2: Telomere melt curve, showing the amplification of a single product

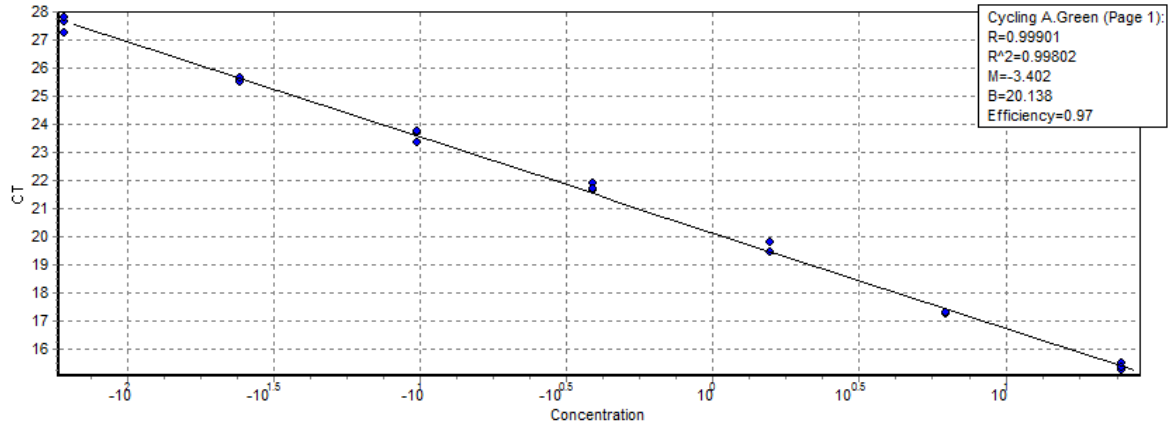


Figure A3: Standard curve for 18S with R^2 = 0.99802 and an efficiency of 0.97

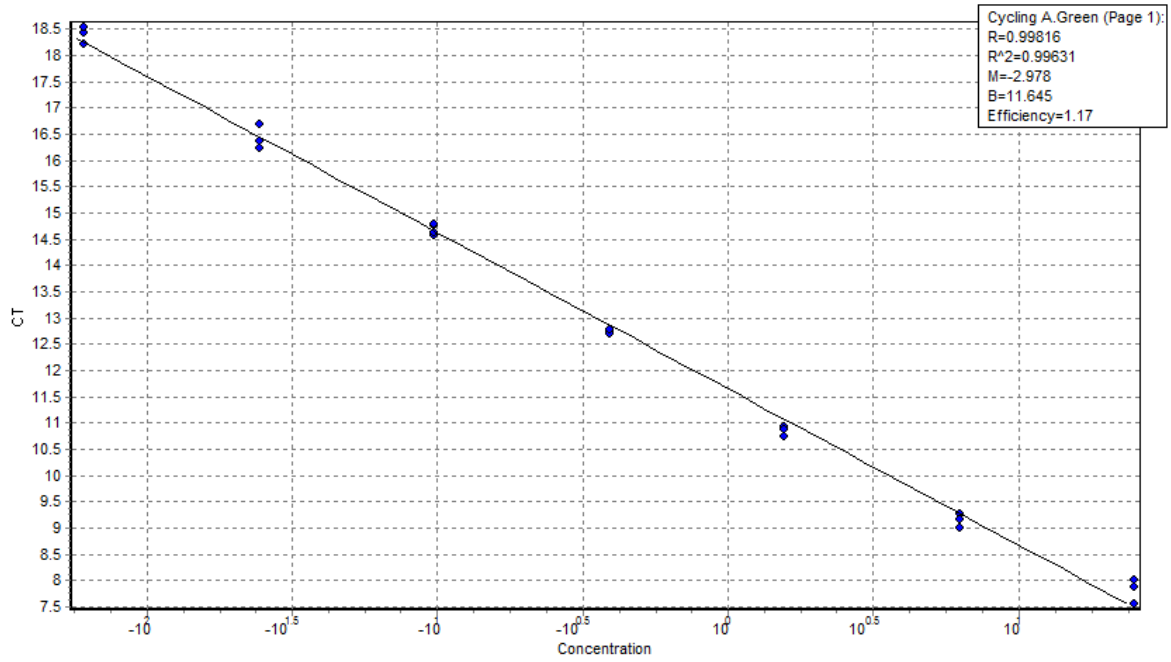


Figure A4: Standard curve for telomeres with R^2 = 0.99631 and an efficiency of 1.17

CHAPTER 3: Covariation in sperm telomere length and sperm velocity in a polymorphic reptile

Manuscript in preparation for submission to Behavioral Ecology and Sociobiology as: Rollings, N., Whittington, C.M., Shine, R., Olsson, M., Friesen, C.R., Covariation in sperm telomere length and sperm velocity in a polymorphic reptile.

Abstract

Telomeres are the DNA-protein caps at the end of chromosomes that have been shown to affect probability of paternity in sperm competition, have (some) heritability, and predict life span early in life in some taxa. Thus, sperm telomere length (STL) may be under selection in both parental and filial generations for several reasons. We therefore investigated STL, sperm number and velocity in the painted dragon lizard, *Ctenophorus pictus*, an agamid lizard with distinct male morphs and associated reproductive tactics. We measured sperm and blood telomere length of 26 males and noted their head colour (red, orange, yellow, or blue) and whether or not they had a yellow gular bib. Previous research has reported that yellow males outcompete red males in sperm competition, but we found no significant difference in their STL. By contrast, the STLs of orange males were approximately 50% longer than those of males of the other three head colours and males with bibs had greater STLs than did males without bibs. STL correlated negatively with two measures of sperm velocity, with no significant difference between the morphs in sperm number within an ejaculate. When standardised by head colour or bib to reduce morph-specific variation, STL was weakly but significantly correlated with telomere length in blood. Our research indicates the importance of considering both somatic and gametic telomeres when assessing the interaction between telomere dynamics and life history strategies.

Keywords: *Ctenophorus pictus*, painted dragon lizard, life history, alternative reproductive tactics, blood telomeres, qPCR

Introduction

Telomeres are short tandem repeats (typically TTAGGG) located at the ends of chromosomes (Blackburn 2005; Blackburn and Gall 1978) that prevent the erosion of coding genes, chromosomal fusion and their attrition act as a measure of cellular damage, triggering senescence at a critical minimum (Houben et al. 2008; Hug and Lingner 2006; von Zglinicki 2002). Telomere length (TL) is often a heritable trait (Njajou et al. 2007; Nordfjall et al. 2005; Olsson et al. 2011a; reviewed in Olsson et al. 2018c) and can predict lifespan (Heidinger et al. 2012; Olsson et al. 2011a), and probability of paternity (Pauliny et al. 2018). Given these potential effects associated with telomere length in a male and his offspring, sperm telomere length (STL) is associated with male fitness and should be under selection both in the paternal generation and in his offspring.

Telomere dynamics of the germline are influenced by similar processes as the telomeres of somatic cells in that they shorten during cellular replication due to the "end replication problem" but they may also be lengthened through activity of the enzyme telomerase (Hug and Lingner 2006; Reig-Viader et al. 2016). Telomerase activity is higher in germline tissue compared with somatic cells where telomerase is usually supressed as a guard against tumour formation and cancer (Olsson et al. 2018c). In mammals, telomerase is expressed through the successive stages of spermatogenesis but tapers off in spermatids when telomeres are longest, and ceases in mature spermatozoa (reviewed in Reig-Viader et al. 2016; Wright et al. 1996).

Telomeres may be damaged and thus shortened by reactive oxygen species (ROS; a common by-product of mitochondrial energy production, Beckman and Ames 1998; von Zglinicki 2002) but ROS damage may be averted with endogenous or exogenous antioxidants (Houben et al. 2008; Magwere et al. 2006; Selman et al. 2012). Reactive oxygen species may damage DNA greatly, accelerating telomeric attrition (Monaghan

2010). Mature spermatozoa do not undergo cellular division and have DNA which is highly compacted, compared with most somatic cells (Pudney 1995; Rathke et al. 2014; Zalenskaya et al. 2000). However, spermatozoa and the DNA they carry may be damaged by ROS while in male storage organs (Gasparini et al. 2017; Pizzari et al. 2008), especially when males are exposed to stressful social and environmental conditions (e.g., Hurley et al. 2018; Mora et al. 2017) and this may affect offspring fitness (Boniewska-Bernacka et al. 2018; Sales et al. 2018). As such, multiple mechanisms exist to reduce ROS damage. Sperm are capable of base excision repair, the removal of a damaged DNA base which leaves the backbone of the DNA intact, and full repair of the DNA may potentially be completed by the repair machinery in the egg after fertilisation has occurred (Aitken et al. 2014). Antioxidant levels in the seminal fluid of humans and some other species often correlate with sperm number, performance and decreased DNA damage (Almbro et al. 2011; Bui et al. 2018; Rojas Mora et al. 2017). Importantly these effects may be condition-dependent and may affect sperm velocity, motility and fertilization success (Kahrl and Cox 2015; Mitre et al. 2004; Rakitin et al. 1999). Male condition-dependence of oxidative status affects sperm quality, and there is increasing evidence that male colouration may reflect antioxidant status (Helfenstein et al. 2010; reviewed in, Svensson and Wong 2011; von Schantz et al. 1999) and that colouration and sperm quality can be correlated (Locatello et al. 2006; Peters et al. 2004; Pitcher et al. 2007; Rowe et al. 2010).

The functional importance of STL for sperm is currently unclear, however research is increasingly finding correlations between STL and other life history traits. Clinicians in assisted reproductive medicine are beginning to consider STL as a marker of sperm quality because DNA damage and shortened telomeres are linked to poor sperm performance, infertility and genome instability in men (Bui et al. 2018; Cariati et al. 2016; Rocca et al. 2016). However, this area has rarely been explored in non-model species especially within

the context of male reproductive strategies with only rare exceptions (Olsson et al. 2011a; Olsson et al. 2018c). We predict that STL is influenced by the male's internal and external environment and moulded by selection during the evolution of reproductive strategies (Monaghan 2014; Monaghan and Haussmann 2006).In species where females mate multiply, generating sperm that can outcompete a rival male's is vital for fitness (Parker 1970; Parker and Pizzari 2010). In humans, sperm number, performance, STL and fertility are all positively correlated (Bui et al. 2018; Cariati et al. 2016; Rocca et al. 2016; Santiso et al. 2010), and developmental symmetry correlates with sperm speed and number (Manning et al. 1998). Sperm performance should be linked with male reproductive success (Firman and Simmons 2010; Fitzpatrick and Lüpold 2014; Møller 1988; Simmons and Fitzpatrick 2012) so STL could have important consequences for postcopulatory sexual selection. However, sperm competition puts males in a double bind. The production of more sperm requires increased metabolic activity and cell divisions in the testes (Gomendio et al. 2011; Parapanov et al. 2008; Tourmente et al. 2011), which may then be accompanied by a concomitant increase in ROS production and adaptations to ameliorate the associated oxidative costs and telomere shortening such as increased antioxidant and telomerase activity (del Barco-Trillo and Roldan 2014; Pizzari et al. 2008; Ribou and Reinhardt 2012; Rocca et al. 2018). Nevertheless, faster sperm may have a higher probability of fertilising eggs and longer STLs of these faster sperm may also produce offspring that are more likely to survive to adulthood, by providing direct benefits in embryo development (Cariati et al. 2016) and survival. Sperm telomere length may also provide indirect genetic benefits to multiply-mating females (Boschetto et al. 2011; Fitzpatrick and Lüpold 2014; Friesen and Olsson 2016; Snook 2005). This argument assumes a positive relationship between STL and sperm quality or performance, as has

been documented in humans but to our knowledge has not yet been explored in any non-mammalian taxa.

As a first step towards filling this gap in understanding, we investigated STL, sperm velocity and sperm number in painted dragon lizards, *Ctenophorus pictus*, an agamid lizard that inhabits semi-arid regions in the southern half of Australia. This species is short lived and approximately 80-90% of the individuals that reach maturity die within a year (Olsson et al. 2007b). Individuals are dormant during winter and emerge in early spring then participating in a single mating season. Males acquire and patrol territories, on which females reside and defend them from other males (Olsson et al. 2007b).

Males are polymorphic with respect to head colour (red, orange, yellow or "blue" – a lack of head colour that makes their blue sides appear predominant, Healey et al. 2007; Olsson et al. 2007a; Olsson et al. 2009b) and the presence or absence of a yellow gular "bib" (Healey and Olsson 2009). These colour traits correspond with alternative reproductive tactics, and their telomere dynamics, as measured in their nucleated blood cells, seem to reflect trade-offs between investments into male aggressiveness and telomere maintenance mediated by oxidative stress (reviewed in Olsson et al. 2018b; Olsson et al. 2018c).

Red males outcompete yellow males in aggressive encounters, and emerge from hibernation earlier (Healey et al. 2007; Olsson et al. 2007a; Olsson et al. 2007b). This tactic seems to be underpinned by red males having higher testosterone (Olsson et al. 2007a), which probably acts as a pro-oxidant (e.g., Alonso-Alvarez et al. 2007; Hoogenboom et al. 2012), potentially increasing oxidative stress in germline cells (Tóthová et al. 2013) and telomere attrition. Indeed, red males have shorter telomeres (Rollings et al. 2017a) than do less aggressive yellow males who copulate faster, have larger testes, and outcompete red males in sperm competition (Olsson et al. 2009b).

Similarly males with bibs have shorter telomeres than do non-bibbed males (Rollings et al. 2017a) and these bibbed males are aggressive and tend to win male-male contests and are more attractive to females (McDiarmid et al. 2017). Energy allocation may explain the short telomeres of bibbed males, as they have higher metabolic rates than do non-bibbed males (Friesen et al. 2017a). In the field, bibbed males lose weight more quickly than do non-bibbed males and the effect is exacerbated when their territories border those of aggressive red males (Healey and Olsson 2009; Olsson et al. 2009a). Thus, body condition and resource allocation (e.g., colour maintenance, protection from oxidative stress Friesen et al. 2017b; Giraudeau et al. 2016) seem to underlie these differences in telomere lengths.

Morph frequencies have fluctuated over time in the population sampled for this study; previously the population was composed of red- and yellow-headed but over the past decade has experienced an increase in orange and blue males (Olsson et al. 2011b), thus we have yet to detail their reproductive tactics. Nevertheless, we know that blue males have the shortest blood telomeres and orange males are intermediate to red and yellow in this respect (Rollings et al. 2017a).

The simplest prediction for STL is that among-morph STL matches blood telomere lengths (BTL) of our previous work. However, telomerase expression in the testes (as has been observed in at least one species of lizard, Alibardi 2015) may affect this relationship. As yellow-headed males outcompete red-headed males in sperm competition, and given the known connection of sperm performance, quality and STL in humans (Santiso et al. 2010), we predict that their STLs are longer. That red-headed males have higher testosterone, which can act as an oxidant, adds further support to this expected scenario. Currently, we know less about the reproductive tactics of orange and blue males, and it is possible that these morphs may have longer STLs. Due to the superior sperm competition of the yellow males, we also predict that they would have more and faster sperm. In the

present study we also investigate the correlation between STL and BTL, allowing us to compare data on STL with our previous work with blood telomeres. Research has not typically considered how telomere dynamics may link reproductive physiology and life history traits and we aim to investigate this relationship.

Methods

Study organism

Mature male dragons (*Ctenophorus pictus*, W. Peters, 1866) were caught in October 2014 by hand or noose at Yathong Nature Reserve, NSW, Australia (145°35'E; 32°35'S) and were brought back to holding facilities at the University of Sydney. Animals were collected under a permit issued by NSW National Parks and Wildlife Service (SL100352) and experiments were conducted in accordance with University of Sydney ethics approval (AEC-2013/6050). At capture, we noted head colour and the presence of a bib. Morph assignment by eye is repeatable within and among researchers in the field (Sinervo and Lively 1996, Olsson et al 2007). The lizards were housed individually in opaque plastic tubs (330×520×360 mm) with sandy substrate and exposed to a 12h light: 12h dark light cycle. They were fed mealworms and crickets, dusted with calcium and multivitamins, to satiation each day and the tubs were misted with water once a day. Heat lamps and ceramic hides allowed the lizards to thermoregulate to their preferred body temperature (Melville and Schulte II 2001). Lizards were housed in multiple rooms for logistical reasons.

Blood collection

On December 16th, 2014, 34 male lizards were weighed (±0.01 g), measured snout-to-vent (SVL; ±1 mm), and body condition was calculated as residuals from a mass-SVL regression. Blood was collected in a capillary tube after gently perforating the *vena*

angularis in the corner of the mouth using a sterile 18 ga. needle. The blood was mixed with heparin, centrifuged at 800 x rcf for 15 minutes and plasma removed. The blood cells were then resuspended in 200 µL of PBS and 1 mL of RNAlater (Sigma Aldrich, Australia) added. Samples were stored at -80°C.

Sperm collection

Subsequent to copulation, semen was collected from the female's cloaca by pulling the ejaculate into a 1mL syringe (Olsson, 2001) preloaded with 100µL Hams F-10 medium (Cat # 99175, Irvine Scientific, Santa Ana, CA, USA; 21 mM HEPES buffer, 4 mM sodium bicarbonate, 1 mM calcium lactate, 0.5 mM magnesium sulfate, 5 mg mL−1 (0.5%) human albumin; e.g., (Friesen et al. 2013; Friesen et al. 2014; Mattson et al. 2007; McDiarmid et al. 2017). Sperm samples were kept at 36±1°C in an incubator until they were analysed using computer-assisted sperm analysis (CASA, see below) ≤ 30 minutes after collection; 35-36°C is the preferred body temperature of this species (Melville and Schulte II 2001). A pilot study (n = 6) showed no significant decline in sperm motility or velocity within 60 minutes, a result that was verified in the full data set presented in this paper (see results below).

Sperm performance - Computer aided sperm analysis (CASA)

The ejaculate was diluted to 1x106/mL with Hams F-10 medium (Cat # 99175, Irvine Scientific, Santa Ana, CA, USA; 21 mM HEPES buffer, 4 mM sodium bicarbonate, 1 mM calcium lactate, 0.5 mM magnesium sulfate, 5 mg mL−1 (0.5%) human albumin) and slowly pipetted (10.0µL) into the chamber of a pre-warmed sperm analysis slide (Hamilton-Thorne 2X-CEL®; Beverly, MA, USA; warmed to 35°C on a slide warmer). Sperm were examined with a phase contrast compound microscope (Nikon E200) using the

4x objective. A digital camera (Basler Aviator AVA1000-100gc) mounted via a 0.70x extension tube, captured videos that were analysed using computer-assisted sperm analysis (CASA; Microptic Sperm Class Analyzer 5.4.0.0 SCA®). Sperm motility was determined for a minimum of 100 sperm per individual. In pilot work, we found it impossible to eliminate all particulates from the ejaculate without negatively affecting sperm motility with excessive washing. Therefore, we modified CASA factory settings to classify a sperm as motile only if its velocity was ≥5 μm/s with no constraints on path straightness. In addition, we visually inspected each sperm track to verify that only sperm were included in the analysis and to confirm accurate tracking of multiple sperm with intersecting paths at the time of analysis. We used the following standard average measures of sperm kinematics data generated by CASA for each sample: straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP).

Sperm counts

A subsample of sperm was homogenized by gentle shaking of the tube and preserved for counts in 3% paraformaldehyde in phosphate buffered saline (pH 7.2). Sperm counts were made in triplicate for each sample on a Petroff-Hausser Counter (cat. # 3900, Hausser Scientific). The sum of the average counts for each of the three replicates was divided by the volume of the counted sample (yielding concentration). This concentration was multiplied by total volume of fluid removed from the female to provide an estimate of to the total number of sperm in each ejaculate.

Quantifying relative telomere length

To analyse the relative telomere lengths of sperm and blood (STL and BTL respectively; relative to the 18S gene) we first purified DNA with a DNeasy Blood and Tissue Kit

(Qiagen, Chadstone, VIC, Australia). The sperm samples were first thawed, centrifuged at 3000 x rcf for 15 minutes and the supernatant removed. For blood, 50 µL of the diluted blood-RNAlater solution was used in the extraction. Extractions proceeded according to the manufacturer's instructions. The protein kinase digestion step was run overnight for both the sperm and blood as the sperm required overnight incubation for complete digestion. RNase A (Qiagen, Chadstone, VIC, Australia) was added at the recommended concentration. See supplementary information for quality control details. Ultimately, 26 sperm samples (head colour: red $n = 6$, orange $n = 7$, yellow $n = 9$, blue $n = 4$, bib: yes $n = 8$, no $n = 18$) were of sufficient quality for use in the study and DNA was extracted from all 26 corresponding blood samples. DNA was stored at -30°C.

Telomere length was measured using real-time quantitative PCR (qPCR) using SensiMix SYBR No-ROX Kit (Bioline, Sydney, NSW, Australia) and a Rotor-gene 6000 thermocycler (Qiagen, Chadstone, VIC, Australia) according to published protocols (Rollings et al. 2017a; Rollings et al. 2017b) using techniques developed by Criscuolo (2009) and Plot (2012) with the 18S ribosomal RNA (18S) gene as the non-variable copy number reference gene. Relative telomere length (RTL) was calculated as the ratio of the telomere to 18S starting concentrations, calculated with LinRegPCR 2016.0 (Heart Failure Research Centre, Amsterdam, the Netherlands, Ruijter et al. 2009; Tuomi et al. 2010). See supplementary information for more details. As RTL is a ratio it is necessarily unitless.

Statistical analysis

Statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA) and SPSS 25.0 (IBM, Armonk, NY, USA). Data was tested for normality using a Shapiro-Wilk test and ln-transformed if needed to conform to normality (with the exception of sperm number for which a square root was generated). Potential effects of body condition (BCI) were assessed by generating the residuals of a regression analysis of mass versus SVL.

Pearson's correlation coefficients were generated for all combinations of STL, SVL, mass, BCI, VAP, VSL, VCL and sperm number. Regression analyses were performed with STL as the dependent variable for VAP, VSL and VCL. Mixed model analyses were performed to investigate the factors affecting STL. Head colour, bib status, and BTL were all included in the initial model as fixed effects with room number included as a random factor. BTL was included in the model for two reasons. Firstly, we know that BTL differs by head colour and bib so by including it in the model we can control for organism-wide effects of these morphs, helping clarify whether the morphs have sperm-specific effects (Rollings et al. 2017a). Secondly, inclusion of another tissue type in the model helps to control for any potential effects of interstitial telomeres, which may obscure trends, as we would expect these to be consistent between the cell types (Foote et al. 2013). All three fixed effects were significant and room was retained in the final model due to a significantly smaller -2 Res Log Likelihood (as shown by a χ^2 test with a single degree of freedom). Post-hoc pairwise comparisons were made between the head colours. As both head colour and bib were significantly related to STL a correlation between STL and BTL was assessed in two ways. TL was standardised by head colour, setting the mean of each morph to zero and the standard deviation to one (Nakagawa et al. 2017). The same standardisation was then performed for the bib trait. Mixed model analyses were performed to assess whether sperm number, VAP, VSL and VCL varied with morph. Head colour, bib and mass were included as fixed factors, mass was included to control for testis size in the analysis of sperm number, and room number included as a random factor. Room number did not result in a significantly smaller -2 Res Log Likelihood in any of the tests and was deleted from the final analyses.

Results

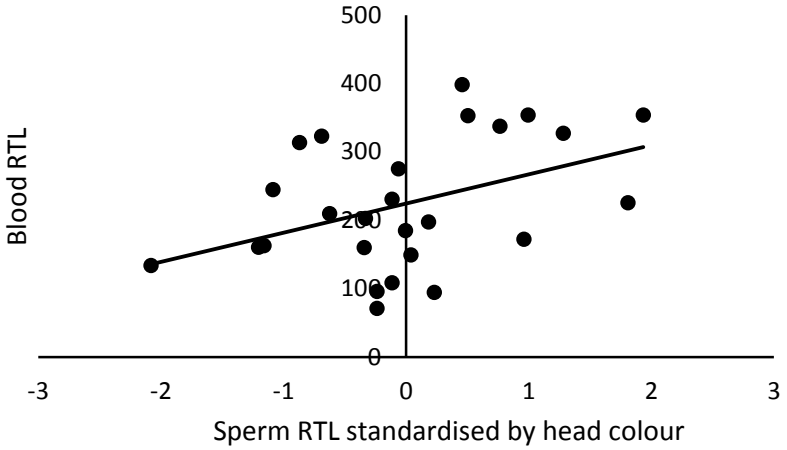
Telomere lengths and physical measures

Mean (±SE) STL was 220.31 (±20.14) and mean BTL was 224.50 (±18.66). Lizard body mass positively correlated with BCI ($r = 0.7459$, $p < 0.0001$, $n = 34$) and SVL ($r = 0.6660$, $p < 0.0001$, $n = 34$). Neither STL nor BTL correlated with mass, BCI or SVL. Sperm telomere length standardised by head colour and bib both had weak positive correlations with BTL (figure 1, head colour: $r = 0.422$, $p = 0.032$, $n = 26$; bib: $r = 0.401$, $p = 0.042$, $n = 26$).

Sperm velocity, number and telomere length

Sperm telomere length negatively correlated with ln-transformed VSL (figure 2a, $r = -0.48795$, $p = 0.0156$, $n = 24$) and ln-transformed VAP (figure 2b, $r = -0.40535$, $p = 0.0494$, $n = 24$). lnVSL, lnVAP and lnVCL were all highly correlated ($r \geq 0.78469$, $p < 0.0001$, $n = 32$). Sperm number correlated with STL but this trend did not attain statistical significance ($r = -0.38121$, $p = 0.0882$, $n = 21$). Sperm number did not correlate significantly with BTL. The morphs did not differ significantly in mean sperm number (Mixed model: $F_{5,23} = 2.008$, $p = 0.115$; head colour: $F_{3,23} = 0.966$, $p = 0.426$; bib: $F_{1,23} = 2.381$, $p = 0.136$; mass: $F_{1,23} = 0.177$, $p = 0.678$) or in lnVAP, lnVCL or lnVSL (head colour: $p \geq 0.295$; bib: $p > 0.099$).

a



b

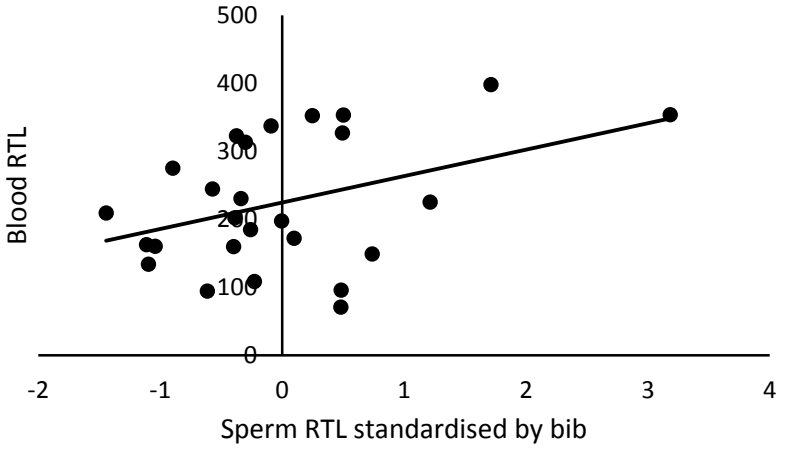


Figure 1: Significant Pearson's correlations between sperm and blood relative telomere length (RTL) a) with sperm RTL standardised (mean = 0, SD = 1) by head colour (r = 0.422, p = 0.032, n = 26) and b) with sperm RTL standardised by bib (r = 0.401, p = 0.42, n = 26).

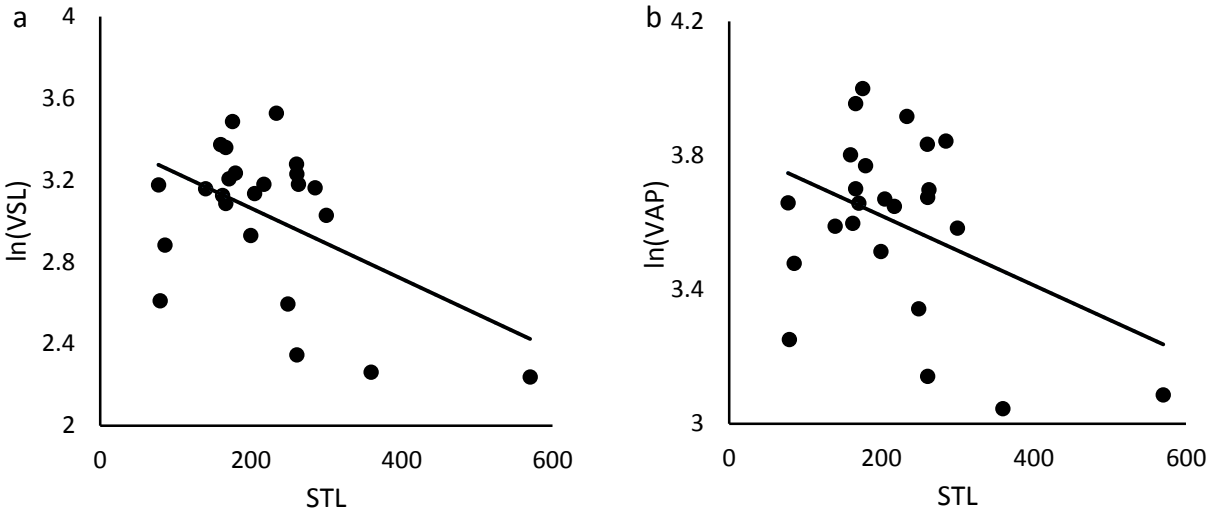


Figure 2: Significant Pearson's correlations between sperm telomere length (STL) and ln-transformed a) straight-line velocity (VSL, r = -0.48795, p = 0.0156, n = 24) and b) average path velocity (VAP, r = -0.40535, p = 0.0494, n = 24).

Morph-specific telomere lengths

Sperm telomere length was significantly correlated with BTL and differed among morphs (Mixed model: $F_{5,18}$ = 5.213, p = 0.004; morph: $F_{3,18}$ = 5.045, p = 0.010; bib: $F_{1,18}$ = 5.138, p = 0.036 ; Linear regression BTL: $F_{1,18}$ = 9.295, p = 0.007). Orange males had the longest mean telomeres (figure 3a, mean ± SE: 294.36 ± 53.90), longer than blue males (176.02 ± 42.50; pairwise contrast: t_{18} = 2.129, p = 0.047), yellow males (203.33 ± 19.86; pairwise contrast: t_{18} = 3.772, p = 0.001) but not red males (188.95 ± 35.04; pairwise contrast: t_{18} = 1.129, p = 0.274). Bibbed males had longer sperm telomeres (figure 3b, 254.55 ± 21.66) than did males without bibs (205.10 ± 27.04).

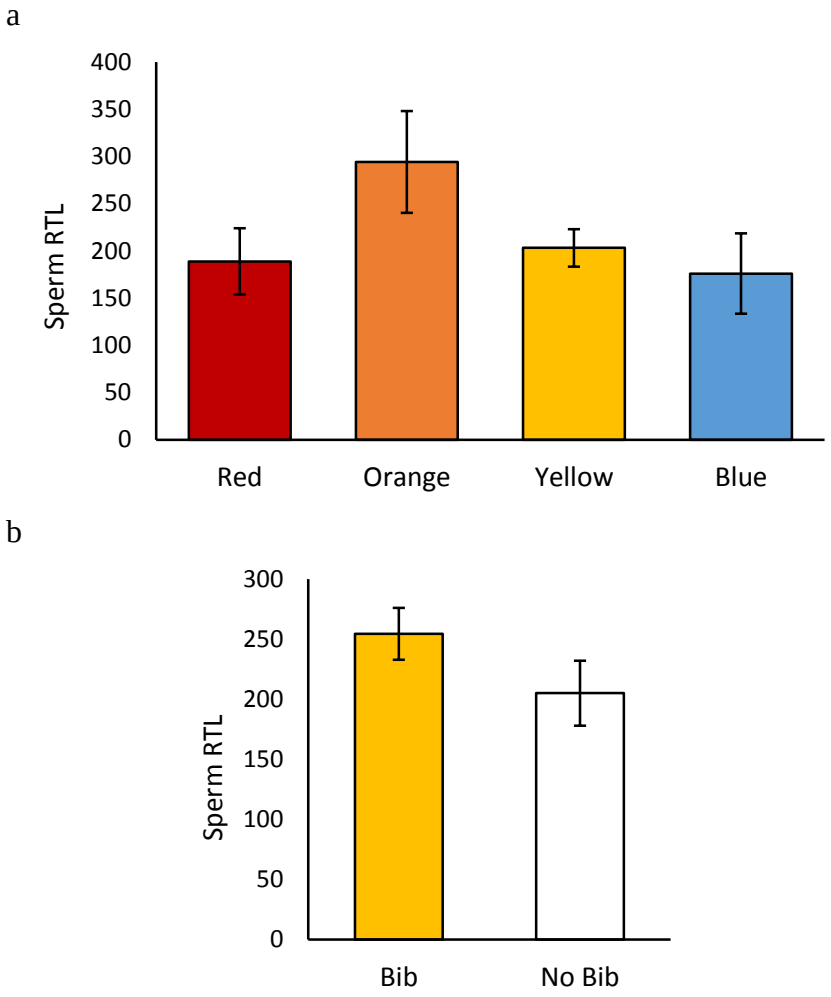


Figure 3: Mean (±SE) sperm relative telomere length (RTL) of the different morph traits: a) head colour (red $n = 6$, orange $n = 7$, yellow $n = 9$, blue $n = 4$), sperm RTL differed significantly between the head colours (Mixed model: $F_{3,18} = 5.045$, $p = 0.010$). Orange males had the longest telomeres, significantly longer than blue males (pairwise contrast: $t_{18} = 2.129$, $p = 0.047$), yellow males (pairwise contrast: $t_{18} = 3.772$, $p = 0.001$) but not red males (pairwise contrast: $t_{18} = 1.129$, $p = 0.274$). b) bib status (yes: $n = 8$, no: $n = 18$), males with bibs had significantly longer sperm telomeres than those without ($F_{1,18} = 5.138$, $p = 0.036$; bib: 254.55 ± 21.66; no bib: 205.10 ± 27.04).

Discussion

The results of the present study indicate that STL may be an important component of the morph-specific reproductive tactics of this species, although the results did not match our original predictions. Orange males had approximately 50% longer sperm telomeres than did males of the other three morphs (the lack of significant difference between the orange and red males is likely due to the small sample size). As previous research on this species has shown that different life history strategies or reproductive tactics may correlate with different telomere dynamics (Rollings et al., 2017), the extended telomeres of the orange males may indicate reproductive tactics unique to this morph. Unfortunately, little is known about the life history strategy of orange males (Olsson et al. 2007a). Visual inspection of orange males suggests a combination of red and yellow pigment, and research that has included red, orange and yellow males has often shown that orange males have behavioural and blood telomere traits that are approximately half way between those of red and yellow males (Healey and Olsson 2009; Rollings et al. 2017a). However the mechanisms that determine head colour appear to be complex, and patterns of inheritance do not support a codominance model whereby a heterozygotic combination of red and yellow alleles lead to orange offspring (Olsson et al. 2007b). Furthermore, previous research shows that orange males have much higher mitochondrial counts in blood samples than the other morphs (Olsson et al. 2018a), suggesting that this morph deviates also in significant biochemistry. Whether these parameters – sperm telomeres and circulating mitochondrial content – are functionally interlinked remains to be investigated.

Longer sperm telomeres are not necessarily adaptive. Orange males may have longer telomeres if their sperm count is lower, due to less cellular division, although in the present study we found no difference between the morphs in sperm numbers within single ejaculates. We observed a negative correlation between STL and sperm velocity,

potentially indicating that longer sperm telomeres decrease sperm competitiveness in painted dragons. Without knowing more about the life history strategy of the orange males, we cannot be certain of the function of STL in this species.

We observed no significant difference in STL between the red and yellow morphs, despite yellow dragons producing sperm that is more successful in competitive fertilization trials (Olsson et al. 2009b). The lack of difference suggests that STL is not necessarily related to sperm competition in this species. At present we do not know what traits improve sperm competition in painted dragons, but further assessment of sperm quality measures such as motility may clarify the mechanisms that lead to the superior success in sperm competition of the yellow morph. One such avenue of research would be whether the higher mitochondrial content in orange males compromises sperm life span more through higher ROS than in, e.g., yellow males.

Bibbed males had longer sperm telomeres than did males without bibs. In order to generate longer telomeres, telomerase activity and potentially antioxidant production in the testes must be upregulated. This may partially explain the higher resting metabolic rate observed in this morph (Friesen et al. 2017a). Increasing STL therefore requires resources which may cause a trade-off whereby less resources are available for somatic maintenance (indeed ROS concentrations in this species predict telomere length, Olsson et al. 2018a), resulting in greater loss in body condition when stressed and, as a corollary, shorter blood telomeres. It is important to note that the lizards were fed ad libitum in the lab, and the mass loss previously documented occurred in the field potentially combined with the higher metabolic rate of bibbed males (potentially combined with the higher metabolic rate of bibbed males: Friesen et al. 2017a; Healey and Olsson 2009; Olsson et al. 2009a), thus the unlimited foraging opportunities in the lab may mask a condition-dependent trade-off.

Sperm storage occurs in this species and females may use stored sperm for multiple clutches (Olsson et al. 2007c; Olsson et al. 2009b). If STL in this species reflects overall sperm quality, this may affect the reproductive tactics of bibbed males. While bibbed males are more prone to losing body condition, if they produce sperm with longer telomeres these sperm may remain viable for longer in the female reproductive tract as telomere length may correlate with overall genome stability. A loss in body condition may increase the probability of dying, but a bibbed male may sire more offspring posthumously if his sperm survives in the female reproductive tract (Olsson et al. 2009b).

In order to test among these potential explanations, further research is required. We need to determine whether STL correlates with offspring telomere length and whether hatchling telomere length can predict survival in this species. The extent to which telomerase, antioxidant and ROS activity in the testes affects STL must also be determined. Due to the great variation in life history strategy between the morphs, assessment of each characteristic needs to be performed at a morph-specific level.

We observed a weak correlation between STL and BTL. This is not unexpected given the difference in trends in STL and morphs compared with our previous morph-based BTL research in which yellow headed males and those without bibs had the longest telomeres (Rollings et al. 2017a). Our results indicate the importance of considering somatic and gametic cells when attempting to clarify the interactions between life history strategies and telomere dynamics.

Ours is one of the first studies to examine correlates of sperm telomere length in a non-mammalian, polymorphic species, and much more remains to be done. Telomere research in non-model species has focused on survival consequences of variation in telomere traits, with less attention paid to the fact that sperm (and egg) telomere biology can link life history traits directly to reproductive physiology. Relative fitness is

determined by *both* survival and reproduction. In previous studies of telomere biology, the focus of research has been on survival-related traits rather than reproduction-related traits. If we are to understand the role of telomeres and their relationship to intraspecific variation in life-history tactics, we need to take a more holistic approach.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Compliance with ethical standards

Animals were collected under a permit issued by NSW National Parks and Wildlife Service (SL100352) and experiments were conducted in accordance with University of Sydney ethics approval (AEC-2013/6050).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary materials and methods

DNA extraction quality control

The DNA concentration (ng μL^{-1}) and A260:A280 ratio of each sample was measured in duplicate using a Pherastar FS (BMG, LabTech, Germany) and aliquots diluted to 10 ng μL^{-1} using the AE buffer provided in the DNA extraction kit. Only samples with an A260:A280 ratio between 1.7 and 1.9 and a concentration above 10 ng μL^{-1} were considered high enough quality to be used in analyses. Where samples had a ratio outside of the desired range purification was attempted. Briefly, sodium acetate was added to the DNA solution to a concentration of 0.3 M. 100% EtOH was added to triple the volume and the mixture placed at -30°C for 40 minutes, followed by centrifugation at 25000 x rcf for 30 minutes at 4°C. The supernatant was removed and 70% EtOH added followed by gentle shaking. The solution was then centrifuged at 25000 x rcf for 15 minutes at 4°C, the supernatant removed and remaining pellet allowed to air dry for up to 30 minutes. The pellet was then dissolved in the provided AE buffer and the A260:A280 ratio measured again. Ultimately 26 sperm samples were of sufficient quality for use in the study. DNA was stored at -30°C.

qPCR methodological details

Telomere length was measured using real-time quantitative PCR (qPCR) using SensiMix SYBR No-ROX Kit (Bioline, Sydney, NSW, Australia) and a Rotor-gene 6000 thermocycler (Qiagen, Chadstone, VIC, Australia) according to published protocols (Rollings et al., 2017a, Rollings et al., 2017b) using techniques developed by Criscuolo (2009) and Plot (2012) with the 18S ribosomal RNA (18S) gene as the non-variable copy number reference gene. A melt curve was generated after each run over the temperature range of 60 to 95°C to ensure that there was no non-specific product amplification, as

evidenced by a single peak. Both of the sperm and blood samples for a given individual were included in the same run. No-template control reactions were run in triplicate for each primer set during every qPCR run to ensure that there was no contamination. Standard curves were produced, using the blood of a randomly selected lizard, for both telomeres and 18S using four-fold serial dilutions to ensure consistent rates of amplification over a wide range of concentrations (26.67 ng μL^{-1} down to 0.037 ng μL^{-1} with 7 different concentrations in total) giving a linear dynamic range of 0.037 - 26.67 ng μL^{-1}. The reaction was considered consistent when the linear correlation coefficient exceeded 0.985. The efficiency of the telomere amplification was 1.05 and the efficiency of the 18S amplification was 0.96, and samples all fell within the same concentration range as our standards. All runs included the same 'golden standard' and also a no-template control to detect contamination. LinRegPCR 2016.0 (Heart Failure Research Centre, Amsterdam, the Netherlands, Ruijter et al., 2009, Tuomi et al., 2010) was used to analyse the qPCR data. The starting concentrations of telomere (T) and control gene (S; 18S) as determined with LinRegPCR were used to determine the relative telomere length with the calculation T/S. Telomeres and the control gene were assessed in separate runs. The mean inter-assay coefficient of variation for qPCR runs for telomere (n = 5) and 18S (n = 6) amplification were 11.04% and 17.47%, respectively, calculated using the golden standard. The intra-assay coefficient of variation for telomere and 18S runs was 21.05% and 9.871%, respectively.

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CHAPTER 4: Telomere dynamics in a lizard with morph-specific reproductive investment and self-maintenance

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Abstract

Telomeres in human fibroblasts shorten progressively during in vitro culturing and trigger replicative senescence. Furthermore, shortened telomeres can be used as biomarkers of disease. These observations have led to the suggestion that telomere dynamics may also be associated with viability and selection for life history variation in non-human taxa. Model systems to examine this suggestion would particularly benefit from the coexistence of multiple phenotypes within the same species with different life history trade-offs, since those could be compared in terms of telomere characteristics. This scenario also provokes the classic question of why one morph does not have marginally higher fitness and replaces the others. One explanation is that different morphs have different reproductive tactics with equal relative fitness. In Australian painted dragons (*Ctenophorus pictus*), males differ in head color, the presence or absence of a gular bib, and reproductive expenditure. Red males out-compete yellow males in dominance contests, while yellow males copulate quickly and have higher success in sperm competition than red males. Males with bibs better defend partners against rival matings, at the cost of loss of body condition. We show that yellow-headed and bib-less males have longer telomeres than red, blue and bibbed males, suggesting that telomere length is positively associated with higher investment into self-maintenance and less reproductive expenditure.

Keywords: alternative reproductive tactics, *Ctenophorus pictus*, painted dragon lizard, TA-65, trade-offs

Introduction

Telomeres are tandem nucleotide repeats (TTAGGG in most metazoans, Gomes, Shay, & Wright, 2010) found at the ends of chromosomes (Blackburn & Gall, 1978; Hug & Lingner, 2006) that can lengthen or shorten during an organism's life in response to stress and ageing (Harley, Futcher, & Greider, 1990; Liu, 2014). These nucleotide repeats are involved in several important functions, such as the triggering of cellular, and possibly, tissue-and whole-organism senescence (von Zglinicki, 2002).

Furthermore, telomeres ensure coding DNA is not lost when linear DNA is replicated (the end replication problem, Hug & Lingner, 2006), prevent the accidental fusion of chromosomes by the repair machinery of the cell (through tight binding with proteins, Houben, Moonen, van Schooten, & Hageman, 2008), and are required for the correct alignment and separation of chromosomes during cell division (Lin, Smith, & Blackburn, 2004). These processes are costly and telomeres become shorter over the life of an organism (at least in homeotherms) due to repeated cellular replication and damage caused by reactive oxygen species (ROS, Chen, Hales, & Ozanne, 2007), which is often elevated during stress (Ludlow, Spangenburg, Chin, Cheng, & Roth, 2014). Another contributing factor to the age-dependent shortening of telomeres may be that up-regulation of telomerase production carries costs traded off against other self-maintenance processes. In addition, increased testosterone levels (or reduced levels of estrogen) may increase telomere attrition and explain sex differences in longevity (males have shorter lives, Barrett & Richardson, 2011) because telomere shortening may trigger cellular senescence (Valko et al., 2007). Given the costs and benefits (sometimes substantial) of "long telomeres," we therefore expect that classic life history trade-offs are mediated by, or reflected in, telomere dynamics and should vary across morphs with different reproductive tactics.

Balanced polymorphism occurs when individuals within a population exhibit different phenotypes that coexist over evolutionary time. This phenomenon has been an intriguing puzzle in evolutionary ecology for generations (Darwin, 1871; Gross, 1996; Richman, 2000), begging the question why does not one morph (with the marginally higher fitness) replace the others? There are multiple mechanisms that may maintain polymorphism, such as when heterozygous individuals have greater fitness than their homozygous counterparts (Richman, 2000). Alternatively, the relative fitness of a morph may be frequency-dependent, decreasing as the morph's frequency within a population increases (Gross, 1996). An example of frequency-dependent selection is the common side-blotched lizard (*Uta stansburiana*) in which orange-, blue-, and yellow-throated males compete against each other in a classic "rock-paper- scissors" game: each morph has a fitness advantage in competition against one morph but is out-competed by a third morph (Sinervo & Lively, 1996). The highly dominant orange males maintain large territories containing many females but cannot effectively guard these females from sneak copulations by yellow, female mimics (Sinervo & Lively, 1996). Blue males effectively guard against the yellow males but are displaced by orange males. As yellow males specialize in sneaking copulations, they are likely to always face sperm competition and their sperms are under selection for long-term sperm storage, which results in posthumous paternity (Zamudio & Sinervo, 2000). Interestingly, the aggressive orange males have higher testosterone and shorter lives than yellow sneaker males (Zamudio & Sinervo, 2000).

Phenotypic differences remarkably similar to those of the side-blotched lizard also occur in the Australian painted dragon lizard (*Ctenophorus pictus*). Males have distinct color-based strategies with differences in somatic self-maintenance and reproductive expenditure, which predicts among-morph differences in telomere attrition without having

to account for phylogenetic differences associated with among-species comparisons of telomere dynamics (since the morphs, largely, share the same genome). Male *C. pictus* are head and gular color-polymorphic, with individuals having red, orange, yellow, or no ("blue") head coloration. All males have blue coloration on their body sides. Most research has focused on the red and yellow morphs, since orange and blue morphs have only started appearing in the population in 1997. The yellow coloration is carotenoid-based, while the red is likely pteridine-based (Olsson, Healey, Wapstra et al., 2007). Red males emerge earlier post-hibernation (Olsson, Healey, Wapstra et al., 2007), experience a greater increase in testosterone throughout the day than yellow males, and outcompete yellow males in dominance contests (Healey, Uller, & Olsson, 2007; Olsson, Healey, & Astheimer, 2007). Conversely, yellow males have larger testes and four times higher reproductive success in sperm competition trials than red males, despite much shorter copulations (Olsson, Schwartz, Uller, & Healey, 2009). This suggests that these two morphs exhibit alternative reproductive tactics that likely result from fundamental differences in trade-offs of resources between testes, testosterone-driven aggression, and longevity. Additionally, male dragons possess another polymorphic feature: a yellow, probably carotenoid-based, gular bib (Healey & Olsson, 2009). The presence of a bib identifies more short-term reproductively successful males, producing more single paternity clutches, at a substantially higher loss in body condition due to mate defense than bib-less males (Olsson, Healey, Wapstra, & Uller, 2009). Thus, polymorphisms could offer, for the first time, explanations of intraspecific variation in telomeres among individuals that share most of their genome but differ in reproductive tactics and expenditure. The variation in reproduction and self-maintenance trade-offs among the morphs is likely to stem from among-morph differences at a cellular level, and manifest in differences in age-and growth-related telomeres.

In order to better understand the underlying role of telomeres in the mediation of life history biology, we used a two-pronged approach. Given their differences in reproductive expenditure, somatic maintenance, growth, ageing, stress, and elevated testosterone levels, telomere dynamics are predicted to vary among morphs. We therefore compared telomere lengths among morphs of *C. pictus*. We also attempted to directly manipulate telomere length through an increase in telomerase activity using TA-65, a herbal supplement that is purported to function as a telomerase activator (Bernardes de Jesus et al., 2011) by up-regulating telomerase transcription factors which bind to the activation domain of the telomerase gene and up-regulate transcription (Valko et al., 2007). This manipulation unfortunately had no effect and is reported in Appendix I to minimize the interruption of the text.

Methods

We quantified the telomere lengths of mature male painted dragons (*Ctenophorus pictus*) of four different head color morphs (Figure 1). The dragons are ideal models for this research as they are short-lived and age rapidly (surviving ~1 year in the wild, Olsson, Healey, Wapstra et al., 2007), making it more likely that attempts to manipulate telomeres will be detected (Olsson, Tobler, Healey, Perrin, & Wilson, 2012).

This work was performed under the Animal Ethics permit 2013/6050 at the University of Sydney. Mature (~9 months old) male lizards were caught by noose or hand at Yathong Nature Reserve, NSW, Australia (145°35′E; 32°35′S) and taken to holding facilities at the University of Sydney in October 2014 where they were housed for the duration of the experiments. Adult males were housed individually in opaque plastic tubs (330 × 520 × 360 mm) with sandy substrate and exposed to a 12-h light: 12-h dark light cycle.

Figure 1: Plates of the four different male painted dragon morphs (*Ctenophorus pictus*)

The males were housed in three different rooms for logistical reasons, but morphs were randomly distributed among rooms. The lizards were fed mealworms and crickets, dusted with calcium and multivitamins, to satiation every day and the cages were misted with water once a day. Heat lamps and ceramic hides were provided to allow the lizards to thermoregulate to their preferred body temperature (36°C; M.O., unpublished data obtained from cloacal temperature readings in the wild).

Blood sampling

Blood (~150 µl) was sampled using a capillary tube prior to and after the completion of the treatment by rupturing the *vena angularis* (in the corner of the mouth) with the tip of a syringe. Collected blood was mixed with heparin and centrifuged, the plasma removed and the remaining cells resuspended with 200 µl of PBS. One milliliter of RNAlater (Sigma

Aldrich, Australia) was added and the diluted blood cell solution for qPCR stored immediately at −80°C.

Quantifying telomere length: qPCR

To analyze telomere length, we first purified DNA from 50 µl of the using a DNeasy Blood and Tissue Kit (Qiagen, Australia), according to the manufacturer's instructions. RNase A (Qiagen, Australia) was added at the recommended concentration. The DNA concentration (ng/µl) of each sample was measured in duplicate using a Pherastar FS (BMG, Labtech, Germany) and aliquots diluted to 10 ng/µl using the AE buffer provided in the DNA extraction kit. Samples were then stored at −30°C. Telomere length was measured using real-time quantitative PCR (qPCR) using SensiMix SYBR No-ROX Kit (Bioline, Sydney, Australia). The telomere primers used were Telb1 (5′-CGGTTTGTTTGGGTTTG GGTTTGGGTTTGGGTTTGGGTT-3′) and Telb2 (5′-GGCTTGCCTTA CCCTTACCCTTACCCTTACCCTTACCCT-3′) (Criscuolo et al., 2009). The 18S ribosomal RNA (18S) gene (92 bp amplicon in *Anolis*) was selected as the reference gene as it had previously been validated in a reptile (Plot, Criscuolo, Zahn, & Georges, 2012). The primer sequences used were 18S-F (5′-GAGGTGAAATTCTTGGACCGG- 3′) and 18S-R (5′-CGAACCTCCGACTTTCGTTCT- 3′). The melt curves produced for both telomere and 18S after amplification by qPCR displayed a single peak, indicating specific amplification of the DNA sequence. The qPCR was performed in a final volume of 20 µl for both telomeres and 18S. DNA of 10 ng was used per reaction, and the primers were used at a concentration of 250 nM. SensiMix SYBR No-ROX Master Mix (Bioline, Australia) of 11.25 µl was added per reaction and $MgCl_2$ was added for a reaction concentration of 1.7 mM. Reactions were run in triplicate for each sample. Amplifications were carried out in a Rotor-Gene 6000 thermocycler (Qiagen, Australia) using an initial

Taq activation step at 95°C for 10 min, and a total of 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s. A melt curve was created after each run over the temperature range of 60–95°C to ensure no non-specific product amplification. No-template control reactions were run in triplicate for each primer set during every qPCR run to ensure no contamination. Standard curves were created, using the blood of a randomly selected lizard, for both telomeres and 18S to ensure consistent rates of amplification over a wide range of DNA concentrations. The reaction was considered consistent when a straight line with an R^2 exceeding 0.985 could be fitted to the values obtained. Threefold serial dilutions were created, starting at a concentration of 26.67 ng/µl down to 0.037 ng/µl, with seven different concentrations in total (Supplemental Figure S1) giving a linear dynamic range of 0.037–26.67 ng/µl. The efficiency of the telomere amplification was 1.05 and the efficiency of the 18S amplification was 0.96. All samples fell within the concentration range generated by the standard curve. In all runs, the no-template controls had a Cq value at least 10 times higher than the lowest sample measured, indicating that contaminant DNA made up a maximum of approximately 0.001% of the original DNA concentration. LinRegPCR 2015.2 (Ruijter et al., 2009; Tuomi, Voorbraak, Jones, & Ruijter, 2010) was used to analyze the qPCR data. LinRegPCR calculates individual starting concentrations based on the average efficiency of an amplicon, the baseline fluorescence, and the threshold cycle values. The starting concentrations of telomere (T) and control gene (S; 18S) were used to determine the relative telomere length with the calculation T/S. Telomeres and the control gene were assessed in separate runs. The mean inter-assay coefficients of variation for qPCR runs for telomere (n = 13) and 18S (n = 13) amplification were 16.73% and 38.21%, respectively, calculated using a reference sample at 10 ng/µl that was included in all runs. Due to the high inter-assay coefficient for the 18S runs, we checked individual values and found an outlier which inflated the inter-assay

coefficient. When this outlier was removed, the mean inter-assay coefficient was 23.52%. To test whether this value was more reliable, we calculated a new mean inter-assay coefficient based on a standard from the standard curve that was included in all runs at a concentration of 8.89 ng/µl. Under these conditions, the mean inter-assay coefficient was 22.30%. This suggests that the actual mean inter-assay coefficient for 18S was approximately 23%. The mean (±SD) intra-assay coefficients of variation for telomere and 18S runs were 14.63 ± 0.1% and 12.52 ± 0.09%, respectively. Mean (±SD) amplification efficiencies generated by LinRegPCR across telomere and 18S qPCR runs were 1.89 ± 0.04 and 1.98 ± 0.01, respectively. LinRegPCR efficiency values can be compared with the efficiency obtained by a standard curve by subtracting 1. The difference in efficiency between the standard curve and LinRegPCR method is likely due to the manual setting of the Cq in the standard curve method.

Statistical analysis

For analysis of morph-specific effects on telomere length, the qPCR data were first entered into a mixed model analysis in Proc MIXED SAS 9.4 (SAS Institute) using telomere length in February (end date of experiment) as the response variable, the predictor fixed factors morph (yellow, orange, red, and blue), and bib (bibbed and not bibbed), and with the room in which a lizard was held (numbered 1–3) as a random factor. When "room" was not significant in a likelihood ratio test ($\chi^2 < 1$, $p > .9$), we performed the corresponding analysis in Proc GLM (in order to obtain R^2 values for our analyses). Telomere length in December was used as a covariate, which controlled telomere length at the onset of the experimental period. This also constrains the analysis to non-interstitial telomeres, since it effectively measures the change in telomere length (under the assumption that interstitial telomeres do not change during the experimental period). That said, we do not suggest that

interstitial telomeres are irrelevant to viability and other fitness effects under selection in association with telomere dynamics. The data obtained from this experiment will be archived in Dryad.

Results

In accordance with predictions, yellow-headed males (n = 27) had telomeres that were significantly longer than both red (n = 20) and blue males (n = 17, Table 1 and Figure 2), whereas orange-headed males (n = 9) were intermediate between yellow and red males, as previously demonstrated for some condition-dependent traits in this species (Healey & Olsson, 2009). Our prediction that bibbed males (n = 24) with more costly reproductive investments should have shorter telomeres was also upheld; males without bibs (n = 52) had telomeres 1.5 times longer than those of bibbed males (Figure 3). There was no significant interaction between head color and presence of bib. Telomere length in February was also significantly predicted by telomere length at the onset of the experiment in December (Table 1).

Table 1: Mixed model analysis of the interactions of telomeres in February with morph type and bib presence in painted dragons, *Ctenophorus pictus*.

Solution for Fixed Effects							
Effect	**morph**	**bib**	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**
Intercept			-0.03734	0.1563	24.3	-0.24	0.8132
morph	**BLUE**		-0.5438	0.1850	66.7	-2.94	0.0045
morph	**ORANGE**		-0.08845	0.2328	63.4	-0.38	0.7052
morph	**RED**		-0.4009	0.1796	67	-2.23	0.0290
morph	**YELLOW**		0	.	.	.	.
bib		**NO**	0.4162	0.1560	66.4	2.67	0.0096
bib		**YES**	0	.	.	.	.
TelDec			0.6859	0.07890	67	8.69	<.0001

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
morph	3	65.2	3.57	0.0186
bib	1	66.4	7.12	0.0096

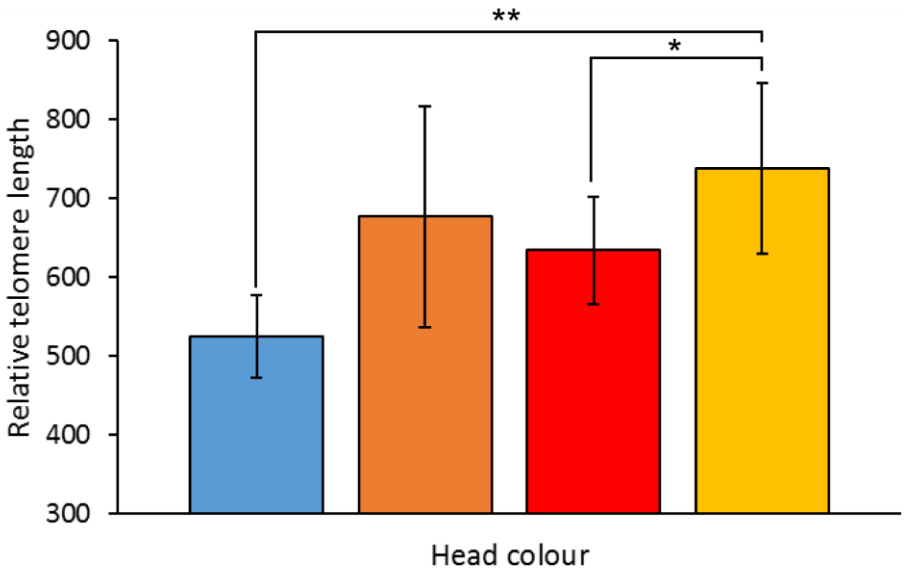

Figure 2: Mean (±SE) relative telomere lengths (RTL) of the different morphs from blood sampled in February. RTL of yellow males was significantly higher than red and blue males (see Table 1).

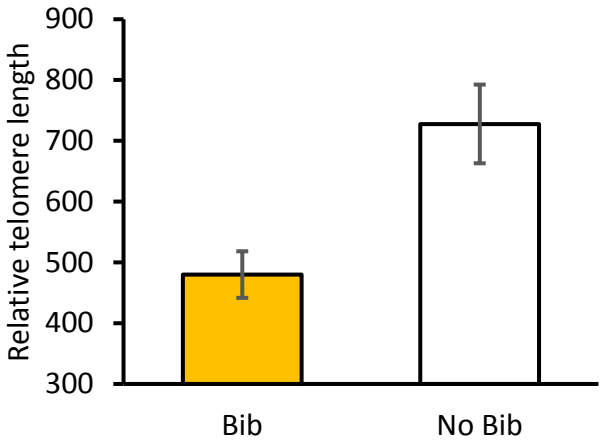

Figure 3: Mean (±SE) relative telomere lengths of males with and without bibs in blood sampled in February (at the end of the experiment). Bibbed males had significantly shorter telomeres than males without bibs ($p = .0096$).

Discussion

This study provides the first evidence that individuals with different reproductive and life history tactics of the same species have different telomere dynamics. Red and yellow males have well-established strategies described in our previous work, which provides context for the morph-dependent telomere attrition. The telomeres of red males were shorter than those of yellow males, potentially revealing a cost associated with red males' higher levels of testosterone and earlier reproductive activities in spring, such as territory establishment. In yellow males, on the other hand, we would predict lower testosterone-dependent metabolic rate and lower ROS levels, resulting in less telomere attrition. The shorter telomeres of the bibbed males provided additional support for the hypothesis that cellular maintenance is traded off against costs of greater, short-term reproductive success (Olsson,

Healey et al., 2009) and that the phenotypic trade-off between these traits differ among morphs.

The results obtained for the orange and blue males are intriguing but, as their reproductive behavior and strategies have not been explicitly investigated, the implications of their telomere dynamics are less straightforward. While not significantly different, the telomere length of the orange males was numerically intermediate to the red and yellow males, suggesting that orange males are heterozygotes of red and yellow alleles. Although previous research indicates that the system of color inheritance is more complex (Olsson, Healey, Wapstra et al., 2007), the life history strategy of orange males may still be some intermediate to that of red and yellow males. Blue males had the shortest telomeres and this may indicate higher levels of ROS (perhaps as a result of lower levels of antioxidants). It is tempting to speculate that pigments (e.g., carotenoids) are used to counter damaging ROS effects, leading to the lack of head coloration observed in blue males. However, blue males are still capable of producing bibs. While the exact composition of the yellow bib pigment is unknown, it may contain both carotenoids and pteridines, indicating that ROS levels are not sufficiently high to remove all pigment-based coloration. Furthermore, this interpretation is further complicated by our experimental demonstration that carotenoid intake has no depressing effect on ROS levels (Olsson, Wilson, Isaksson, Uller, & Mott, 2008). In order to understand the mechanisms behind the telomere dynamics of these two morphs, additional studies of their life history and reproductive strategies are required.

To conclude, our results demonstrate that telomeres, polymorphisms, and life history strategies are strongly interlinked. We have observed differences in telomere dynamics across not just one, but two different types of polymorphisms within a single species, revealing telomeres to capture the trade-offs at a cellular level caused by alternative reproductive tactics.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Appendix I

Based on the results in Reichert et al. (2014), we predicted that TA-65 would have telomere-enhancing effects across all morphs and that telomeres would be shorter in high-investment strategies (thus predicting relatively shorter telomeres in red and bibbed morphs). We therefore assessed whether TA-65 had the capacity to alter telomere length, possibly through the amount of telomerase activity and telomere length. Given its oral administration, this would be a non-invasive, direct manipulation of telomere length and its repercussions (Bernardes de Jesus et al., 2011; Reichert et al., 2014). Males were weighed to the nearest 0.01 g and snout-to- vent and total length were measured to the nearest 1.0 mm prior to commencing the TA-65 treatment. In addition, head color and presence of a bib were recorded and the males were randomly assigned to the TA-65 treatment (n = 41) or control (n = 35) groups. Males were treated each weekday for 7 weeks, across January and February, with 0.7 mg of TA-65 (T.A. Sciences, USA), dissolved in 100 µl of saline, given orally. This dosage was selected as an increase in telomere length was observed when zebra finches were treated with 0.5 mg per 100 µl of water (Reichert et al., 2014). The dosage was increased by 40% to account for the decrease in body temperature and metabolic rate experienced by reptiles at night. The TA-65 solution was prepared each day just prior to administration. Control males were treated identically but given only saline. TA-65 did not significantly affect telomere length ($p > .40$ in all analyses) and was backwards-eliminated from all analyses reported on in this manuscript. Future studies of telomere dynamics should ideally include telomere manipulation experiments. Although telomerase knock-out organisms have been a successful tool for studying telomere dynamics (Anchelin et al., 2013; Mourkioti et al., 2013), the difficulty and cost of generating knock-outs limit our ability to investigate telomeres in non-model organisms. A compound that could be orally administered would be ideal for these purposes; however,

our experiments showed no effect of TA-65 on lizard telomere length. There are a few possible explanations for this: (1) Typical telomerase activity in painted dragons may maintain telomeres at their maximum stable length for each morph; thus, higher telomerase activity may not increase telomeres further. (2) Previous studies have focused on endotherms and thus the dosage chosen may not have been appropriate for an ectotherm (although in the current experiment the bird dose (Reichert et al., 2014) was increased by 40% to compensate for the fall in nightly body temperature to ambient levels in an ectotherm). (3) The telomerase activator needs to be able to bind to the activation domain of the telomerase gene, and if the activation domain and telomerase activator are not appropriately matched, binding may not occur, nullifying the desired manipulation. Only future dose–response effects across a wider range of concentrations will clarify the utility of this compound for potential future telomere manipulation work in ectotherms.

Supplementary Information

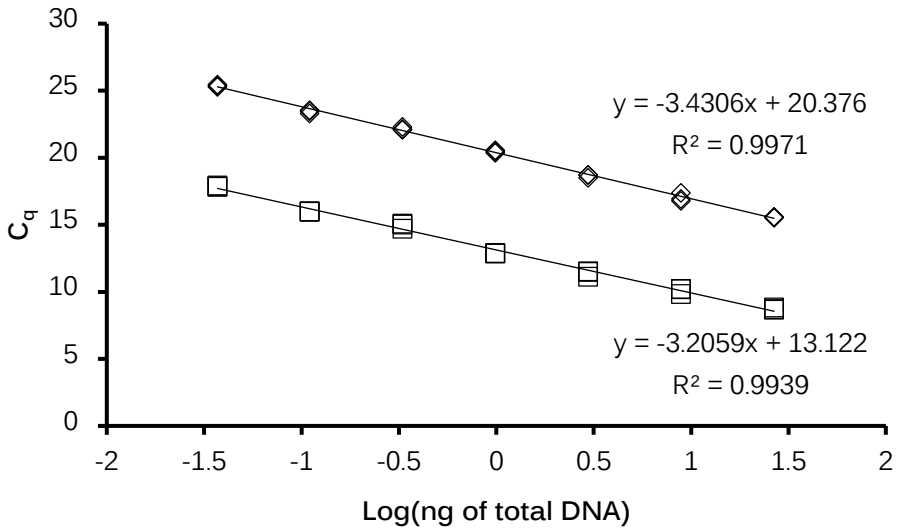

Figure S1: Standard curves were generated to determine the consistency of the qPCR reactions over a wide range of concentrations. Diamonds represent the reference gene 18S and squares represent telomeres.

CHAPTER 5: Age-related sex differences in body condition and telomere dynamics of red-sided garter snakes

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Abstract

Life-history strategies vary dramatically between the sexes, which may drive divergence in sex-specific senescence and mortality rates. Telomeres are tandem nucleotide repeats that protect the ends of chromosomes from erosion during cell division. Telomeres have been implicated in senescence and mortality because they tend to shorten with stress, growth and age. We investigated age-specific telomere length in female and male red-sided garter snakes, *Thamnophis sirtalis parietalis*. We hypothesized that age-specific telomere length would differ between males and females given their divergent reproductive strategies. Male garter snakes emerge from hibernation with high levels of corticosterone, which facilitates energy mobilization to fuel mate-searching, courtship and mating behaviours during a two to four week aphagous breeding period at the den site. Conversely, females remain at the dens for only about 4 days and seem to invest more energy in growth and cellular maintenance, as they usually reproduce biennially. As male investment in reproduction involves a yearly bout of physiologically stressful activities, while females prioritize self-maintenance, we predicted male snakes would experience more age-specific telomere loss than females. We investigated this prediction using skeletochronology to determine the ages of individuals and qPCR to determine telomere length in a cross-sectional study. For both sexes, telomere length was positively related to body condition. Telomere length decreased with age in male garter snakes, but remained stable in female snakes. There was no correlation between telomere length and growth in either sex, suggesting that our results are a consequence of divergent selection on life histories of males and females. Different selection on the sexes may be the physiological consequence of the sexual dimorphism and mating system dynamics displayed by this species.

Keywords: telomeres, condition, life-history strategies, sex-differences, reptile

Introduction

Life-history strategies vary widely both between and within species. Such strategies describe how limited resources are used and prioritized [1,2], generating trade-offs between different physiological processes that mediate growth, reproduction and survival [3–5]. For example, organisms that 'live fast' are characterized by rapid growth and maturation, and high reproductive output, but age more quickly and have short lifespans [6,7]. Conversely, organisms that 'live slow' grow and mature more gradually and have lower reproductive output, but age more slowly and have longer lifespans [6,7]. Reproduction–longevity trade-offs are often difficult to detect within a population due to condition-mediated positive correlations between natural history traits [4,8]. However, there should be a link between condition, cellular maintenance and ageing. Body condition reflects the efficient collection, assimilation and deployment of resources and depends on the individual's capacity to cope with handicaps like infection, injury, parasitism and environmental stress throughout ontogeny [9–14].

As long-lived organisms age, they tend to experience reduced survival and reproductive output that may be mediated by condition ([15], but see, [16]).One mechanism linking differences in life histories, lifespans and ageing appears to be variation in telomere dynamics [17–19]. Telomeres are hexameric tandem repeat sequences of 5'-TTAGGG-3' at the ends of chromosomes that typically shorten over the life of an organism due to repeated cellular divisions and damage caused by reactive oxygen species (ROS; [20,21]). Among species, telomere dynamics may covary with life-history strategies [22,23], and the rate of telomere attrition correlates with lifespan [17,24]. However, it is unclear whether short telomeres cause death or whether they are correlated with some other mechanism of senescence [18,25,26]. Body condition indices (BCI: body mass

controlled for structural length) may be a useful measurement of somatic maintenance that is associated with longer telomeres (e.g. [18]).

Interspecific differences in telomere attrition are probably due to prioritizing cellular maintenance (e.g. DNA-repair) over other cellular functions [27,28], as autosomal mutations are negatively correlated with lifespan (mammals, [29]). DNA damage can lead to mutations, telomere loss and cellular senescence; thus, the maintenance of the genome probably explains telomere length stability in longer lived organisms [30,31]. To date, most studies of telomere dynamics and life history strategies have focused on interspecific comparisons [17,19,22,25]. While these studies have yielded insight into telomere dynamics, elucidating the mechanisms underlying the observed trends is complicated by genetic variation between species. Studying organisms that exhibit intraspecific differences in reproductive tactics and/or life-history strategies provides a natural experimental scenario to study telomere dynamics while minimizing the noise of interspecific genetic variation. For example, females and males often exhibit sex-related differences in reproductive strategies and sexual selection [32–36], which may result in sex-specific telomere dynamics [24,37–39]. Thus, we sought to investigate telomere dynamics in a highly dimorphic species with well-characterized life history and reproductive strategies: the red-sided garter snake, *Thamnophis sirtalis parietalis* (a non-venomous colubrid).

Red-sided garter snakes are sexually dimorphic with respect to body size, with females growing approximately 30% longer, on average, than males [40]. In the Interlake populations of Manitoba, Canada, red-sided garter snakes hibernate for eight months in communal dens and emerge *en masse* in spring, to form large aggregations where males scramble to locate and mate with females [41,42]. Mating activity at the dens lasts approximately six weeks from late April through May [43] with some males mate-searching and courting for two to four weeks [41,44,45]. During the spring breeding

season, male garter snakes are aphagous and have relatively high levels of corticosterone [46–50].Courtship and copulatory plug production are energetically expensive [42,51,52], and males may lose 10% of body mass during two weeks of mate-searching, courtship and mating [44,50]. In other species, physiological stress and fasting lead to increased ROS production, the depletion of endogenous antioxidants, and increased cellular damage and senescence [53–61]. One of the hallmarks of male ageing is poor sperm performance, which is strongly influenced by oxidative and other physiological stressors (reviewed in [62]). Indeed, larger (and therefore older, [63]) male red-sided garter snakes have poorer sperm performance than smaller males [64], suggesting that these males undergo senescence in the wild. In contrast with males, female garter snakes seem to prioritize growth and maintenance over short-term reproductive success. Females reach sexual maturity at 3 years of age, while males are sexually mature at 1 or 2 years [65]. Most females mate every year before migrating to feeding grounds [66], but they reproduce only when they have acquired sufficient body mass or 'capital', which is typically every other year [67,68]. Like most snakes, female garter snakes do not provide post-natal parental care [69]. Furthermore, female fecundity increases with body length [70–72] and, presumably, also with age because snakes exhibit indeterminate growth [63]. Biennial reproduction and increasing reproductive fitness with age may generate selection on increased cellular maintenance, body condition and growth in females. In this species, body condition is positively correlated with fat mass (Uhrig et al. 2012, unpublished data). With such life-history variation between the sexes, the red-sided garter snake is an exceptional model for investigating how different reproductive strategies and telomere dynamics interact, while minimizing the genetic variance that makes interspecific studies difficult to interpret. We hypothesized that the sex-specific reproductive strategies of red-sided garter snakes would be associated with differences in age-related declines in

telomere lengths. This study aims to determine: (i) the relationship between body condition, telomere length and age in garter snakes, and (ii) whether this relationship differs with sex. We predict that male garter snakes will experience greater telomeric attrition with age than females, due to the much more intense reproductive investment in males. Furthermore, if females are investing more in somatic maintenance than males, we expect females will maintain better body condition.

Materials and Methods

At the peak of breeding season (10 May 2015),we collected an excess of snakes by hand from mating aggregations with the aim to collect the full range of body lengths found at our Inwood, Manitoba, study site (males: N = 100; females: N = 50). We transported snakes to Chatfield research station, 16 km away, where they were weighed (± 0.01 g) and measured for snout–vent length (SVL: ± 1 mm) where we culled our sample to ensure an equal distribution of sizes for each sex. We selected the four longest and four shortest animals of each sex and an even distribution of intermediate sizes, obtaining a final sample of 42 males and 30 females (figure 1*a*), the remaining 78 animals were returned to the point of capture the next day. All animals were adults; juveniles are only rarely found at den sites (R.T.M. > 25 years of personal observation; [41,70]). Blood (less than 0.1 µl) for telomere analysis was taken from the caudal vein, added to 300 ml of RNAlater and frozen (-30°C) until DNA extraction. Approximately 1 cm of tail tissue was collected for skeletochronological ageing; see expanded methods in electronic supplementary material for more details.

Skeletochronology/histology

Individual age was estimated by a modified version of the technique described by Waye & Gregory ([74,75] 1999) and Clesson, Bautista [76]. Vertebrae were examined microscopically and the number of growth rings was identified for each animal; see electronic supplementary material, for more details.

Quantifying telomere length

Telomere length was measured using real-time quantitative PCR (qPCR) as we have done previously [77] using the 18S ribosomal RNA (18S) gene as the non-variable in copy number reference gene [77–79]; see electronic supplementary material more details.

Statistical analysis

We calculated two measures of BCI. In both cases BCI is the standardized residuals (mean = 0; s.d. = 1) from linear regressions of ln(body mass) as a function of ln(SVL) [80]. We ran this linear regression model once with males and females pooled, and it was clear that females had much higher BCI than males. Therefore, it was more biologically relevant to generate BCI for each sex separately using a separate regression model for each sex, thus creating BCI specific for each sex (ssBCI) to account for differences in allometry [80]. Growth was calculated as size (SVL)/age. Visual inspection of regression plots for male telomere length given age suggested a curvilinear relationship as has been described in many taxa, including squamate reptiles [25,38,81–84], and F-tests we used to formally test the goodness of fit for first-order versus quadratic regressions. We used ANCOVA to test for age-specific sex differences in telomere length and body condition. When we found a significant sex by age interaction we used the Johnson–Neyman (J-N) procedure to determine ages where the sexes differed in condition [73]. All analyses were conducted in

SigmaPlot 13.0, except the J-N procedure which was conducted in MS Excel on the spreadsheet provided as electronic supplementary material in White [73]. See electronic supplementary material, for more details.

Results

Skeletochronology, size and body condition

Age and sex predicted body size (SVL): older animals were longer and females were significantly longer than males of the same age (ANCOVA: sex × age p = 0.487 (dropped from model): $R^2 = 0.366$; age: $F_{1,69} = 14.636$, $p < 0.001$; sex: $F_{1,69} = 16.569$, $p < 0.001$; figure 1*a*). The shape of the age distributions was not different between the sexes (Kolmogorov–Smirnov test: $D = 0.205$, $p = 0.412$) and females in our sample were significantly older than males ($F_{1,70} = 6.384$, $p = 0.014$; mean (range), females: 4.3 years (2–9 years); males: 3.5 years (2–6 years)). There was a significant sex × age interaction on BCI (ANCOVA: $R^2 = 0.542$; age: $F_{1,69} = 5.403$, $p = 0.023$; sex: $F_{1,69} = 0.003$, $p = 0.953$; sex × age $F_{1,69} = 8.695$, $p = 0.004$), suggesting that females and males differentially maintain body condition as they age. Because of the significant sex age interaction, we computed the region of non-significance for the age-effect on BCI between the sexes (28.454 to 2.029 yrs) using the J-N procedure [73]. This approach demonstrates that BCI differed between the sexes at ages greater than 2.03 years, which included most of the snakes in this sample (figure 1*b*; note age values < 0 are meaningless and omitted from the figure). Given the profound sex-differences in body condition, we recalculated BCI for each sex with separate regressions (i.e. ‘sex-specific’ BCI) and reran the analysis. We still found a significant sex × age interaction ($p = 0.023$), which revealed that sex-specific body condition tends to increase with age in females, but decreases with age in males (electronic

supplementary material, figure S2). We used this sex-specific BCI (ssBCI) to explore the relationship between body condition and telomere length in further analyses.

Telomere length and age

Telomere length was shorter in males than females ($F_{1,70} = 7.288$, $p = 0.009$). The relationship between telomere length and age was different for males and females. Age did not predict telomere length in females (females: simple linear regression $R^2 = 0.000$, $F_{1,29} = 0.005$, $p = 0.945$: quadratic regression; $R^2 = 0.000$, $F_{2,28} = 0.050$, $p = 0.951$; figure 2a). However, in males, telomeres shorten with age, a relationship better fit by quadratic regression than linear regression (test of first-order = null hypothesis versus quadratic: $F_{2,41} = 5.538$, $p = 0.024$: simple linear regression: $R^2 = 0.108$, $F_{1,41} = 4.856$, $p = 0.033$; quadratic regression: $R^2 = 0.219$, $F_{2,39} = 5.472$, $p = 0.008$; figure 2*b*).

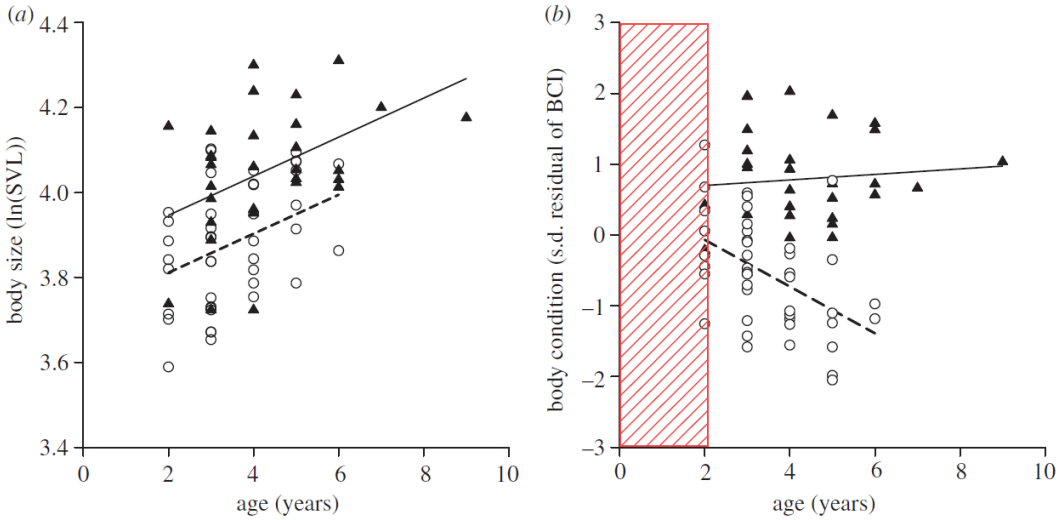

Figure 1. (*a*) Age (years) and sex predicted body size (ln(snout-to-vent length): ln(SVL)): older animals were longer and females were significantly longer than males of the same age. Open circles indicate males and solid triangles indicate females (note: for clarity with overlapping data points, male data are offset slightly to the right). The least-squares regression lines were calculated separately for females (solid line, $r = 0.400$) and males (dashed line, $r = 0.445$). (*b*) Body condition (BCI) differed with age and sex and there was a significant sex × age interaction ($p = 0.004$). Females had higher BCI than males and BCI decreased with male age but not females. Open circles indicate males and solid triangles indicate females. The least-squares regression lines were calculated separately for females (solid line, $r = 0.015$) and males (dashed line, $r = -0.479$). The diagonal hatched box (age = 0.00 to 2.03), is the age-range through which BCI did not differ between females and males as determined by the Johnson–Neyman procedure [73].

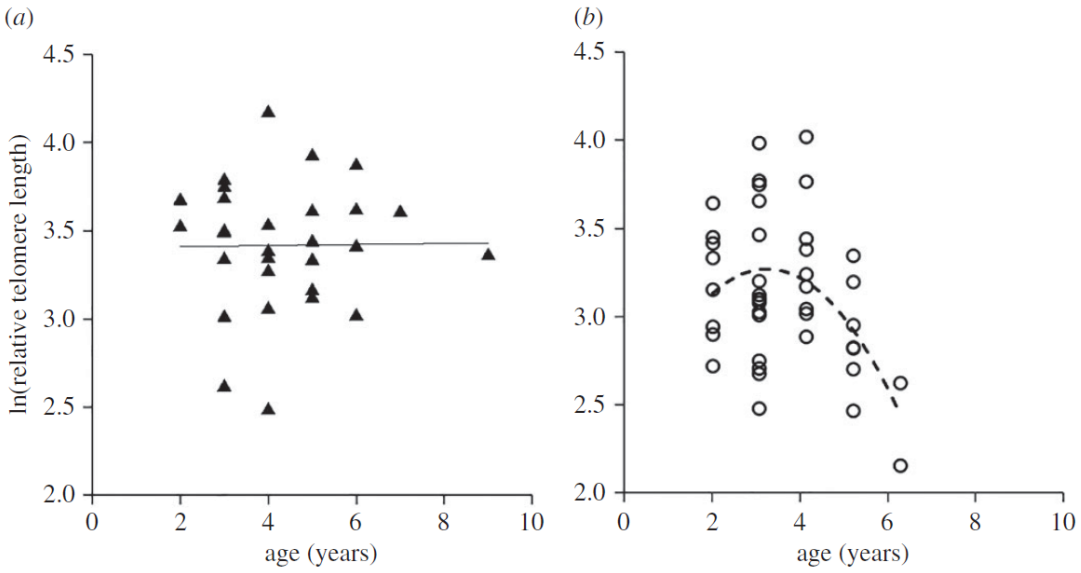

Figure 2. The relationship between natural log of blood telomere length and age in years was different for females (a) and males (b). (a) Age did not predict telomere length in females (females: simple linear regression; $r = 0.013$, $F_{1,29} = 0.005$, $p = 0.945$: quadratic regression; $r = 0.067$, $F_{2,29} = 0.050$, $p = 0.951$). (b) However, in males, telomeres shorten with age, which is better fit by quadratic regression than a linear regression (test of first-order = null hypothesis versus quadratic: $F_{2,41} = 5.538$, $p = 0.024$: quadratic regression: $r = 0.468$, $F_{2,41} = 5.472$, $p = 0.008$).

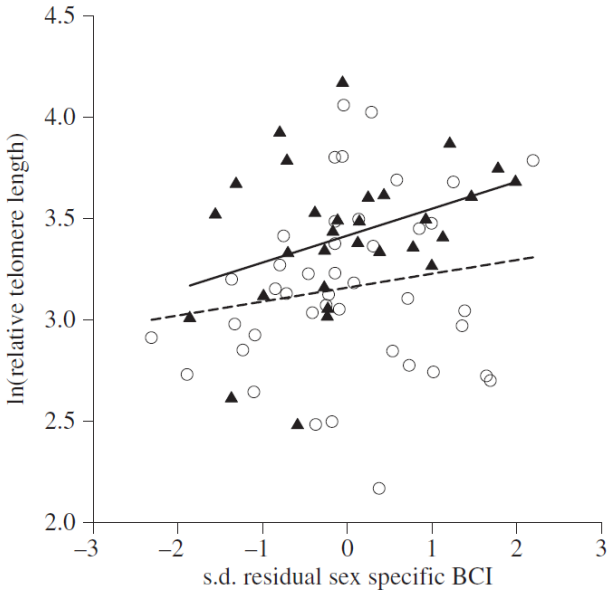

Figure 3. Combined sex-specific body condition (standardized residuals from separate regressions of body mass given snout-to-vent length for each sex) and natural log of blood telomere length were positively correlated (r = 0.602). Females had higher BCI than males, but the relationship between BCI and telomere length was the same. Open circles indicate males, and solid triangles indicate females. The least-squares regression lines were calculated separately for females (solid line, r = 0.362) and males (dashed line, r = 0.506).

Telomere length, body size and growth

Although age and SVL were directly related in both sexes (see above), SVL and telomere length were not related (ANCOVA sex × SVL p = 0.538 (dropped interaction): R^2 = 0.095; sex: $F_{1,69}$ = 5.900, p = 0.018; SVL: $F_{1,69}$ = 0.057, p = 0.813). Separate analyses to test for a quadratic relationship, as was found in males for age and telomere length, showed no evidence for a relationship between SVL and telomere length in either sex (females p = 0.200; males p = 0.229). Finally, growth (size/age) was not significantly associated with telomere length (either SVL/age: R^2 = 0.052, p = 0.085; residual SVL given age: R^2 = 0.031, p = 0.137; or sex-specific residual SVL given age: R^2 = 0.001, p = 0.766).

Telomere length and body condition

Sex-specific body condition (ssBCI) and blood telomere length were positively correlated (R^2 = 0.131, $F_{1,70}$ = 10.564, p = 0.002), and, although females had higher ssBCI than males, the relationship between ssBCI and telomere length was the same for both sexes (ANCOVA sex ssBCI: p = 0.510 (dropped interaction): R^2 = 0.145; sex: $F_{1,69}$ = 7.601, p = 0.007; ssBCI: $F_{1,69}$ = 4.005, p = 0.049; figure 3).

Discussion

Sex-differences in aging may result from sex-specific optimization of investment to reproduction and somatic maintenance in response to the challenges of different life-history strategies between the sexes. We have shown that body condition positively correlates with telomere length in both sexes of red-sided garter snakes, which supports our assertion that body condition is an intuitive measure of somatic investment. However, the relationship between body condition and age differed strikingly between sexes, with females maintaining their body condition with age, while condition decreased with age in males. Likewise, telomeres were exponentially shorter in older male garter snakes, while the telomere lengths of females were independent of age. Nonlinear relationships between telomere length and age have been shown in several taxa (e.g. [81,84]), and is consistent with an exacerbating cycle of cellular damage and increased dysfunction seen in ageing humans [85]. Females had the longest telomeres and were the oldest individuals in our sample, suggesting they live longer than males in this population. These results support our prediction that males experience greater telomere loss with age due to prioritization of current reproduction over cellular maintenance and longevity. Overall, the decrease in both body condition and telomere length in males with age suggests that they senesce at an earlier age than females.

Telomere shortening has been implicated as a cost of reproduction in several species. For example, in blue tits (*Cyanistes caeruleus*), when brood size was experimentally increased, parents experienced a decrease in blood telomere length, with males suffering from greater telomere loss than females [86]. Relative reproductive success seems to result in greater telomere attrition in common terns (*Sterna hirundo*) [87]. For both male and female Atlantic silversides (*Menidia menidia*), gonadal somatic index (GSI: gonad mass relative to total body mass) was negatively correlated with telomere length and lifespan [88]. These studies suggest increased reproductive investment comes at a cost of telomere attrition.

Studies of telomere dynamics are rare in reptiles and there are only two reports on snakes. Bronikowski [89] reported telomere lengths for male wandering garter snakes (*Thamnophis elegans*). Wandering garter snakes are an interesting species for studying telomere dynamics because in the mountains of Northern California there are two eco-types with very different life histories: one short lived 'meadow' eco-type and a long-lived 'lakeshore' eco-type [90–92]. As in our study, Bronikowski [89] showed declining telomere length with male age (up to 12 years of age, based on skeletochronology), but was unable to find among eco-type differences, and did not report telomere lengths for females. In water pythons (*Liasis fuscus*) of Northern Territory, Australia, females have longer telomeres than males [82], similar to our study. Furthermore, telomere length increased from hatching to 4 years of age, but declined very slightly with age in both sexes up to 18 years of age [82].

Why might selection on telomere dynamics differ between male and female garter snakes?

Our study is observational and cross-sectional, so our causal interpretation of the sex-specific differences in the relationship between age and telomere length is necessarily tentative. In Manitoba's Interlake region, winter temperatures often hover around -40°C for weeks and, because snow provides insulation from the cold, there are likely cryptic mass fatalities deep within dens during years of light snowfall [70]. The snakes' brief three to four month active season begins and ends with chance freezes and floods that lead to mass mortality events that are likely to generate selection on rapid growth and early maturity in both sexes [70,93]. Mortality due to these stochastic events is usually not consistently biased toward either sex and the adult sex-ratio is 1:1 [41,70,93]. Predation and road kills are not sex biased either [93]. However, a mass mortality event could differentially affect size classes among sexes. For example, a winterkill event in 1998–1999 shifted the size distribution toward smaller animals in subsequent years in both sexes, but the largest females were most strongly affected [93]. Small males, and to a lesser extent large females, are more likely to be trapped and suffocate in large mating aggregations (more than 500 animals) [94]. Such events could cull a size class or spare only old females with the longest telomeres, generating results similar to ours. Nevertheless, we have not witnessed similar events in our yearly visits since 1999, thus other explanations may better fit our results.

Males engage in energetically expensive reproductive behaviour annually, while most females generally reproduce biennially. Although male size affects mating success when a single pair of males competes for copulation, the effect is small to non-existent in the largest aggregations at the den sites, reducing selection for increased male size [95,96]. The largest females, however, are able to reproduce annually, leading to greater fecundity and generating higher selection on female growth and longevity [70–72,97]. Females in

Interlake populations seem to have higher reproductive output given female size than populations farther south in less harsh climates with longer feeding/growth seasons [70,98]. Therefore, selection on cellular maintenance and longevity are likely to be stronger in females than males because the costly mechanisms that prevent telomere loss are balanced by increasing fecundity with age and size in females, but have fewer benefits for males.

What physiological mechanisms might explain sex-specific telomere attrition?

We do not know the specific mechanisms that lead to sex-differences in telomere length, but there are several non-mutually exclusive hypotheses to explain our results. For these ectotherms, body temperature and metabolic rate are very low during winter brumation (approx. 1°C [99]) and only rise in late April when the ground warms. Both sexes enter winter hibernacula at the same time [70], but males, on average, emerge earlier than most females. Therefore, body temperature and metabolic rate will be lower, for slightly longer, in females than males. Lower body temperature associated with torpor is correlated with positive effects on telomere length and somatic maintenance in some mammals (e.g. [100]).

High levels of corticosterone experienced by males during the mating season [49] may increase metabolism, but also may increase mitochondrial ROS production, DNA damage and telomere erosion [31,56,101,102]. The high energetic demands of courtship and mating of aphagous males [51] probably limits the resources that can be allocated to DNArepair mechanisms, limiting the chance for telomere repair [103]. For example, the increased male–male competition among male rhesus macaques (*Macaca mulatta*) is correlated with DNA oxidative damage (8-OHdG) and shorter lifespan [57]. In red-sided

garter snakes, the energy for antioxidant synthesis, DNA-repair and telomere maintenance is limited by male fasting [27,54,55,59,104]. Fasting itself may increase oxidative stress [53,58,60,61]. Fasting increases the generation of mitochondrial ROS and lipid peroxidation in rats (*Rattus norvegicus*) [61]. Fasting male northern elephant seals (*Mirounga angustirostris*) exhibit increased oxidative damage to DNA and lipids [58]. Given the stochastic mortality, weak sexual selection on male size, and oxidative stress induced by during energetically costly courtship and mating while fasting, selection to mitigate damage by ROS via investment in cellular maintenance and growth may be weak in male red-sided garter snakes. Weak selection for enhanced cellular maintenance might explain both the reduction of body condition and telomere length with age. This may be the consequence of selection for a live fast, die young strategy in males.

Females were in better body condition than males in our study, which generally indicates they have larger energy stores than males [105]. In brown tree snakes, *Boiga irregularis*, and in female red-sided garter snakes, this additional energy reserve correlates with lower levels of corticosterone [106,107], potentially leading to lower stress overall and more stable telomere length [28,108]. Furthermore, having greater energy reserves may allow for greater expenditure on antioxidants and cellular repair. Species of snakes that live longer are capable of producing a stronger response to DNA damage by activating repair mechanisms and experience lower levels of mitochondrial ROS, which presumably generates less oxidative damage to DNA [89,109]. We show that female *T. sirtalis* parietalis have a greater lifespan than males and may potentially use mechanisms similar to those of other snakes to maintain genome stability and telomere length. The underlying mechanisms causing the sexual dimorphism may provide explanations for sex-specific differences in telomere length.

Sexual size dimorphism varies greatly across taxa, and trends associated with dimorphism, lifespan and telomere attrition are not consistent [24,110]. For garter snakes, the difference in size between males and females seems to be controlled by testicular androgens suppressing growth in males [111]. Testosterone can reduce cellular resistance to free radicals [112], leading to increased DNA damage and telomeric attrition [19,113]. In the closely related red-spotted garter snake, *Thamnophis sirtalis concinnus*, females treated with an oestrogen receptor antagonist, tamoxifen, experienced a decrease in growth rate [114], suggesting that oestrogen plays a role in the sexual size dimorphism observed in *T. sirtalis parietalis*. Oestrogens act as antioxidants and/or stimulate endogenous antioxidant and cellular repair mechanisms [115–117] potentially reducing ROS and leading to the telomeric stability observed in this study and in females across other taxa [24,38]. The most energetically demanding component of reproduction for female garter snakes is the production of yolk proteins (i.e. vitellogenesis) [118]. There is evidence that the yolk protein, vitellogenin, may act as an antioxidant, [119–123] reducing DNA damage, telomere attrition and cellular senescence at a time when cellular respiration and ROS production are highest. Thus, selection acting on the mechanisms that increase female growth and provisioning of offspring seem to also favour antioxidant production, a reduction in oxidative stress and cellular repair involved in slowing the ageing process.

In this cross-sectional study, we investigated differences in telomeres within a single species. We found that telomere dynamics is strongly linked with sex and therefore life-history strategies. Sex-specific telomere dynamics may be tightly linked to selection on males for early reproduction and costs associated with yearly energetic investment in courtship and mating while fasting. By contrast, females have biennial reproduction and investment in somatic maintenance has a fitness pay-off of greater fecundity with increasing size later in life. Future studies should include longitudinal data, increased

sampling of the largest size classes, the measurement of telomerase activity, general DNA damage, and antioxidant production throughout the entire active season, to assess our hypothesis that females live longer by investing more in cellular maintenance and repair than males.

Ethics

Procedures performed on animals were approved by Oregon State University (IACUC ACUP-4317) and the research was conducted under permit from Manitoba Conservation (WB16264).

Data accessibility

Data have been uploaded to the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.jv463 [124].

Authors' contributions

All authors have made significant intellectual and material contributions to this paper.

Competing interests

The authors declare they have no competing interests.

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Supplementary Full Materials and methods

Materials and methods

At the peak of breeding season (May 10, 2015), we collected snakes by hand from mating aggregations with the aim to collect the full range of body lengths found at our Inwood, Manitoba study site (males: N = 100; females: N = 50). We transported snakes to Chatfield research station, 16 km away, where they were weighed (±0.01g) and measured for snout-vent length (SVL: ±1mm). To ensure an equal distribution of sizes for each sex, we selected the 4 longest and 4 shortest animals of each sex and an even distribution of intermediate sizes, obtaining a final sample of 42 males and 30 females (see FIGURE 1a), the remaining 78 animals were returned to the point of capture the next day. All animals were adults; juveniles are only rarely found at den sites (RTM > 25 years of pers. obs.; Gregory, 1977, Gregory, 1974). Blood (<0.1mL) for telomere analysis was taken from the caudal vein, added to 300 µL of RNAlater and frozen (-30˚C) until DNA extraction. Prior to collection of tails for skeletochronological aging, the tail of each animal was wiped clean with reptile ringers solution, and then 95% ethanol, before receiving local anaesthesia. We injected 15-µL of 2% Lidocaine HCl subcutaneously and lateral to the vertebrae, 5 mm cranial to the site of tail excision. Ten minutes after Lidocaine injections, the distal 1-1.5cm of the tail was severed using a sterilized, single-edged razor blade on an aseptic field, and styptic powder was applied to the tail. (The 'surgical' field was cleaned with soap and water, then rinsed with 5% bleach solution between excisions. The razor was sterilized between uses: first cleaned with soap and water, rinsed with deionized water, then sterilized in a Germinator ® 500 dry sterilizer at 260ºC for >15 sec). The tail-tip was preserved in 10% formalin in neutral PBS, until histological analysis (see below). All

animals were released at site of capture after two days of observation and appeared in good condition.

Skeletochronology/Histology

Individual age was estimated by a modified version of the technique described by Waye and Gregory (1998, 1999) and Clesson et al. (2002); see expanded methods in supplemental document 1 for more details. Vertebrae were examined microscopically and the number of growth rings was identified for each animal. All animals were examined by two independent evaluators (HLW and RWK) in a blind test and the findings compared. Variation in age assessments (N = 3) were examined a second time by each evaluator and determined by consultation. This consultation consisted of RWK and HLW marking growth lines on photographs of the questionable sample sections to calibrate their readings, and then re-examining the outstanding slides to reach a consensus on a final age.

Quantifying telomere length

To analyse blood-cell telomere length, we first purified DNA from 50 µL of the RNAlater diluted blood samples using a DNeasy Blood and Tissue Kit (Qiagen, Australia), DNA concentration (ng/µL) of each sample was measured in duplicate using a Pherastar FS (BMG, Labtech, Germany). Telomere length was measured using real-time quantitative PCR (qPCR) as we have done previously (Giraudeau et al., 2016, Rollings et al., 2017) using the 18S ribosomal RNA (18S) gene as the non-variable in copy number (NVC) reference gene. The melt curves produced for both telomere and 18S after amplification by qPCR displayed a single peak, indicating specific amplification of the DNA sequence. The 18S gene has been previously used as a NVC gene in reptile telomere studies (Giraudeau et al., 2016, Rollings et al., 2017, Plot et al., 2012). To validate 18S as non-

variable in copy number gene in this species, we compared relative telomere length in 20 randomly chosen individuals (10 males, 10 females) calculated using 18S as a reference gene, with relative telomere length calculated using eukaryotic translation elongation factor 1 alpha 1 (eef1a1) as a reference gene (sense primer 5'-CCTAATTGTTGCTGCTGGTGTT -3'; antisense primer 5'-GTGCTGACTTCTTTGACAATTTCC -3') (the eef1a1 gene has previously been used as a NVC gene in reptiles Rovatsos & Kratochvíl, 2016, Rovatsos et al., 2015). We found a positive, linear relationship between RTL calculated using the two genes ($r = 0.623$, $F_{1,17} = 10.227$, $p = 0.005$), indicating that either eef1a1 or 18S is a suitable NVC gene for use in this species. More critically, when we compared RTL calculated using 18S and eef1a1, we found no significant difference in the linear relationship between males and females (Sex × Telomere-measurement interaction: $F_{1,16} = 0.460$, $p = 0.507$), ruling out a systematic difference in RTL calculation between the sexes that would bias our results.

Reactions were run in triplicate for each sample. Amplifications were carried out in a Rotor-Gene 6000 thermocycler (Qiagen, Australia) using an initial Taq activation step at 95 °C for 10 min and a total of 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A melt curve was generated after each run over the temperature range of 60 to 95 °C to ensure that there was no non-specific product amplification. No-template control reactions were run in triplicate for each primer set during every qPCR run to ensure that there was no contamination. Standard curves were produced, for both telomeres and 18S to ensure consistent rates of amplification over a three-fold serial dilutions (40 ng/µL down to 0.055 ng/µL with 7 different concentrations in total: Supplemental Figure S1) giving a linear dynamic range of 0.055 to 40 ng/µL. The reaction was considered consistent when a straight line with an R^2 exceeding 0.985 could be fitted to the values obtained. The efficiency of the telomere amplification was 1.01 and the efficiency of the 18S

amplification was 0.90, and samples were all within the same concentration range as our standards. All runs included the same ‘golden standard and also the no-template controls which had a quantification cycle (Cq; Bustin et al., 2009) LinRegPCR 2016.0 (Ruijter et al., 2009, Tuomi et al., 2010) was used to analyse the qPCR data. The starting concentrations of telomere (T) and control gene (S; 18S) as determined with LinRegPCR (below) were used to determine the relative telomere length with the calculation T/S. Telomeres and the control gene were assessed in separate runs. The mean inter-assay coefficient of variation for qPCR runs for telomere (n = 4) and 18S (n = 4) amplification were 11.6% and 18.0%, respectively, calculated using a reference sample that was included in all runs. The intra-assay coefficient of variation for telomere and 18S runs was 15.1% and 14.4%, respectively. LinRegPCR generated efficiencies for telomere and 18S qPCR runs were 1.868 and 1.982, equivalent to 86.8% and 98.2%, respectively.

It is currently unknown whether the chromosomes of *T. sirtalis parietalis* contain interstitial telomeres (TTAGGG repeats found elsewhere in the chromosome; Foote et al., 2013) and, thus, whether they might influence the results. However, interstitial telomeres, which can vary in length between individuals, primarily add random noise to telomere measurements making the signal of telomere dynamics harder to detect (Foote et al., 2013, Smith et al., 2011).

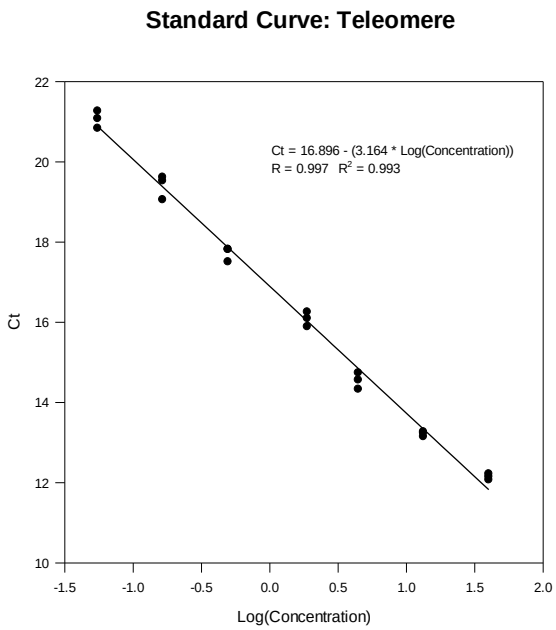
Statistical analyses

We calculated two measures of body condition indices (BCI). In both cases BCI is the standardized (to a mean = 0 and standard deviation of =1) residuals from linear regressions of ln(body mass) as a function of ln(SVL) (Schulte-Hostedde et al., 2005). We ran this linear regression model once with males and females pooled, and it was clear from the pooled sample that females had much higher BCI than males. Therefore, it was more

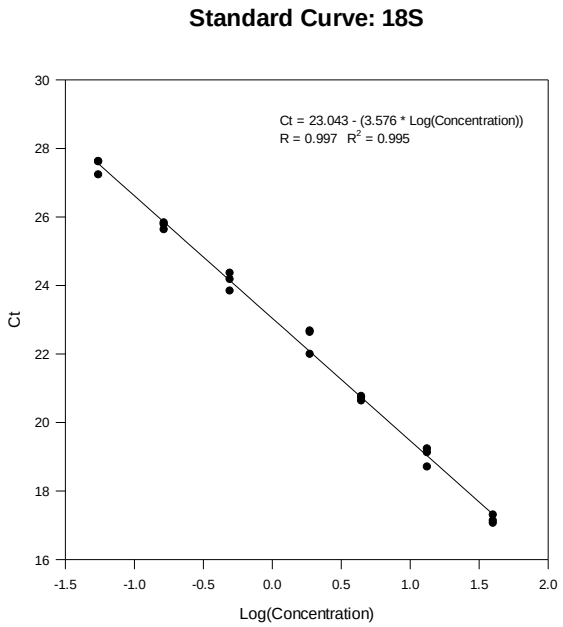
biologically relevant to generate BCI for each sex separately using a separate regression model for each sex with the standardized residuals, thus creating BCI specific for each sex (ssBCI). We calculated growth as the quotient of SVL/age. Residuals of telomere length were not normally distributed when regressed against age, SVL, or BCI. Therefore, telomere length was ln-transformed for all analyses, which corrected non-normality of residual telomere length. We used F-tests using the polynomial regression analysis function in SigmaPlot 13.0 to determine whether telomere length at a given age was better described by first-order or quadratic regression, as visual inspection of the plots seemed to fit a curved line and curvilinear relationship of age and telomere length have been described in many taxa, including squamate reptiles (Ujvari et al., 2016, Ujvari & Madsen, 2009, Anchelin et al., 2011, Haussmann et al., 2003, Olsson et al., 2011, Pauliny et al., 2006). The type or relationship (linear vs curvilinear) between age and telomere length was different between the sexes, so we analysed each sex separately. We used ANCOVA to test for sex differences in telomere length and body condition while accounting for age. Using the methods from White (2003), if there was a significant interaction between sex and the covariate, we used the Johnson-Neyman procedure to determine the region of non-significance between the sexes. All analyses were conducted in SigmaPlot 13.0, except the J-N procedure which was conducted in MS Excel on the spreadsheet provided as a supplement in White (2003).

Standard curves were produced, using the combined blood of five randomly selected snakes, for both telomeres and 18S to ensure consistent rates of amplification over a wide range of DNA concentrations. Three-fold serial dilutions were created starting at a concentration of 40 ng/µL down to 0.055 ng/µL with 7 different concentrations in total (Supplemental Figure S1) giving a linear dynamic range of 0.055 to 40 ng/µL. The reaction was considered consistent when a straight line with an R^2 exceeding 0.985 could

be fitted to the values obtained. The efficiency of the telomere amplification was 1.01 and the efficiency of the 18S amplification was 0.90. All samples fell within the concentration range generated by the standard curve.



Supplemental Figure 1: The telomere qPCR standard curve generated from a pooled DNA sample of four snakes. $R^2 = 0.993$ indicates the reaction proceeds linearly across the tested concentration range.



Supplemental Figure 2: The 18S qPCR standard curve generated from a pooled DNA sample of four snakes. $R^2 = 0.995$ indicates the reaction proceeds linearly across the tested concentration range.

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CHAPTER 6: Sperm telomere length correlates with growth rate in red-sided garter snakes, *Thamnophis sirtalis parietalis*

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Abstract

Telomeres, tandem repeats of TTAGGG at the ends of chromosomes, are highly dynamic structures that shorten in response to a variety of factors, including organismal stress, hormone concentrations, and tissue-specific growth rates. Cell turnover rates are frequently linked to their functions, resource availability and telomere dynamics. Male red-sided garter snakes, *Thamnophis sirtalis parietalis*, are an ideal model for investigating sperm telomere dynamics due to a dissociated reproductive cycle where sperm must remain viable for several months. Males produce sperm in summer and autumn, when resources are abundant, and store it until the stressful spring mating season when they compete for matings against many other males for several weeks. In this study we determined sperm telomere length (STL) and age using qPCR and skeletochronology, respectively. Sperm telomere length correlated positively with snout-vent length (SVL), but not age. Sperm telomere length positively correlated with growth rate proxies (residuals of a size against age regression). Although an individual's STL is correlated with previously measured blood telomeres, blood telomeres are shorter than sperm telomeres, shorten with age in males, and are unrelated to SVL or any growth rate proxies. We hypothesise that STL is related to growth rate and SVL because growth and sperm production both occur during summer when resources are most abundant and stress lowest. This study is the first to compare telomere dynamics between cell types in a reptile, and supports growing evidence that telomere dynamics may be highly tissue-specific and driven by the life history strategy of an organism.

Keywords: telomeres, sperm, growth, ageing, life history, *Thamnophis sirtalis parietalis*, garter snake, reptile

Introduction

Telomeres, short tandem repeats of TTAGGG located at the ends of chromosomes (Blackburn 2000; Blackburn and Gall 1978), have repeatedly been found to correlate with the ageing of an organism (Jaskelioff et al. 2011; Monaghan and Haussmann 2006; Sahin et al. 2011). While longer telomeres allow for greater growth and repair capacity, shorter telomeres limit cellular division, potentially decreasing the risk of cancer but also accelerating the ageing process (Aubert 2014; Monaghan and Haussmann 2006). Telomeres may shorten due to growth and stress (Shalev et al. 2013).

Growth requires cellular division and an increase in metabolism (Brown et al. 2004; Jorgensen 1988), and higher growth rates may lead to greater telomere attrition (Herborn et al. 2014; Pauliny et al. 2015; Tarry-Adkins et al. 2013). Cellular division requires chromosomal replication which results in telomeric shortening due to the 'end-replication problem' (Olovnikov 1973). A higher metabolism may increase reactive oxygen species (ROS) production causing telomeric damage and attrition (Finkel and Holbrook 2000; Houben et al. 2008; Monaghan 2014; Selman et al. 2012; von Zglinicki 2003). However, if resources are sufficient, growth does not always cause telomeric attrition (Young et al. 2017): telomere length can potentially be maintained through the production of antioxidants to counter ROS, or an increase in the activity of telomerase, the enzyme that lengthens telomeres (Giardini et al. 2014; Greider 1990; Monaghan, Metcalfe, Torres 2009).

Hormones may also affect telomere lengths. Stress can result in higher glucocorticoid concentrations, via the hypothalamo–pituitary–adrenocortical axis (Herman and Cullinan 1997). These glucocorticoids typically cause an increase in metabolism and suppression of the immune system (Angelier et al. 2018), which may lead to increased ROS production, reduced antioxidant production, and increased telomeric attrition

(Angelier et al. 2018; Haussmann and Marchetto 2010). Androgens can also negatively affect antioxidant function, with higher concentrations resulting in reduced resistance to ROS (Alonso-Alvarez et al. 2007), potentially increasing telomeric attrition. As such, environmental conditions, food abundance, stress, and hormones are some of the factors that may modulate the telomeric loss a growing organism experiences.

Due to growth and stress, telomeres typically shorten over the life of an organism and are extended shortly after fertilization allowing sufficient telomere length for growth and development (Aubert 2014). One way for this extension to occur is through the production of gametes with longer telomeres, which both increases overall telomere length and may be a guide for telomerase-independent telomere lengthening (de Frutos et al. 2016; Liu et al. 2007; Vizlin-Hodzic et al. 2009; Wright et al. 1996). In several species, sperm telomere length (STL) relates to the telomere length of offspring. The inheritance of paternal telomere length has been observed across a range of taxa, but focussed largely on blood cells (de Frutos et al. 2016; Nordfjall et al. 2005; Olsson et al. 2011). In mice, sire STL influences telomere length in embryos (de Frutos et al. 2016). In humans, STL increases with age (Turner and Hartshorne 2013) and children of older fathers have longer leukocyte telomeres; the heritability of this trait is so high it has even been observed in grandchildren (Eisenberg, Hayes, Kuzawa 2012; Unryn, Cook, Riabowol 2005). As individual and paternal telomere lengths may be predictors for survival (Haussmann, Winkler, Vleck 2005; Noguera, Metcalfe, Monaghan 2018; Olsson et al. 2011; Salomons et al. 2009; Young et al. 2017), and can determine the capacity for repair in an organism (Jaskelioff et al. 2011; Sacco et al. 2010; Wong et al. 2000), sire STL may be an important factor affecting the fitness of successive generations.

Given the potentially critical implications of sire STL for offspring, we assessed sperm telomere dynamics in male red-sided garter snakes, *Thamnophis sirtalis parietalis* of

the Interlake region of Manitoba, Canada. Males in these populations emerge from limestone sinks in the spring after spending 7-8 months >2 meters underground with body temperatures hovering around 0-4˚C (Gregory 1974; Lutterschmidt, LeMaster, Mason 2006). After emergence males search for mates and engage in energetically expensive, competitive courtship behaviour with 10s to 100s of rivals for 2-4 weeks before migrating to feeding grounds many kilometres from the emergence sites (Friesen et al. 2015; Friesen, Powers, Mason 2017a; Gregory and Stewart 1975; Shine et al. 2001). During this period males are aphagic and experience high levels of corticosterone that aides mobilization of fat and protein (Cease, Lutterschmidt, Mason 2007; Krohmer, Grassman, Crews 1987; O'Donnell, Shine, Mason 2004). The risk and intensity of sperm competition is high in this mating system, in part because females can store sperm for many months before fertilization (Halpert, Garstka, Crews 1982). Males have more sperm that are longer than a congener's sperm with less spatially and temporally condensed mating periods (Friesen et al. 2017b). Despite the fact males deposit a large, gelatinous copulatory plug that occludes and seals the female's cloaca (Devine 1975), which acts as temporary passive mate guarding device (Shine, Olsson, Mason 2000) and prevents sperm leakage or ejection (Friesen et al., 2013; Friesen et al., 2016; Friesen et al., 2014d), 80% of litters exhibit multiple paternity (2-4 sires per litter, Friesen, Kerns, Mason 2014a; Friesen et al. 2014b). Friesen et al. (2015) estimated that the production of a single copulatory plug represents up to 18% of the daily energy budget of an actively courting male. The enduring the energetic costs of mate searching, courtship, and copulatory plug production while fasting may come at a cost of reduced life span and DNA damage in the form of telomere erosion (Rollings et al. 2017b). Crucially, males suffer and energetic opportunity-cost given that staying at emergence sites for 2-4 weeks means they miss ~20% time available for foraging before early season freezes forces them back deep underground for the winter. However, males

have evolved a strategy that may reduce the exposure of their sperm to these assaults during spermatogenesis.

Male garter snakes are unusual in that they experience a dissociated reproductive cycle (Crews and Garstka 1982). They produce sperm in late summer through to autumn, a time of low stress and high testosterone, when they also undergo growth and when resources are most abundant (Krohmer et al. 1987). This sperm is then stored over winter for at emergence in the spring (Gregory 1974; Krohmer et al. 1987). As growth and sperm production occur at the same time, we would predict a relationship between the two, namely that an individual with sufficient resources would experience greater growth and also be able to offset potential ROS damage, producing sperm with longer telomeres. However, during the mating season their testes are regressed and inactive, experiencing very little cellular division (Crews et al. 1984; Krohmer et al. 1987). Due to the testicular regression and inactivity during the spring, we would expect that STL would be largely unaffected by the stress of the mating season and thus that STL would not be related to the age of the individual, in contrast to telomeres in blood (Rollings et al. 2017b). We compare the results of the present study with our previously published data on blood telomere length from the same individuals in order to determine similarities and differences between cell types (Rollings et al. 2017b).

Methods

Animal Collection

Procedures performed on animals were approved by Oregon State University (IACUC ACUP-4317) and the research was conducted under permit from Manitoba Conservation (WB16264). At the peak of breeding season, on May 10, 2015, we deliberately chose 100 male snakes by hand from mating aggregations with the aim to collect the full range of body lengths found at our Inwood, Manitoba study site. We transported them to Chatfield research station, 16 km away, where they were held in housed separately in outdoor nylon arenas (1m ×1m × 1m) and were provided water ad libitum. The snakes were weighed (±0.01g) and snout-vent length was measured (SVL: ±1mm). We wanted an equal distribution of age classes (age and length are highly correlated Rollings et al. 2017b), so we selected the 4 longest and 4 shortest animals and an even distribution of sizes in between to obtain a final sample of 42 males. All animals were adults; juveniles are only rarely found at den sites (RTM > 25 years of pers. obs.; Gregory 1974; Gregory 1977). Blood and tail tips, for skeletochronology, were collected according to previously published protocols (Rollings et al. 2017b), see supplementary material for more information.

Copulatory plug collection

Over the course of three consecutive days of mating trials in May 2015, we collected copulatory plugs and the sperm embedded within them from female garter snakes immediately after termination of copulation, as in our previous work on ejaculate investment, sexual conflict and sperm competition (Friesen et al. 2013; Friesen et al. 2016; Friesen et al. 2014d). All males were allowed unlimited access to newly emerged, unmated females that were caught the day before each of three consecutive days of mating trials.

Males were able to mate with multiple females, and thus we obtained a total of 63 copulatory plugs from 41 males, with more than one plug for 26 of the males. In such instances, the copulatory plugs for each male were then assigned a number in order of their insemination and subsequent collection. We placed the plugs in pre-weighed 1.5 mL microcentrifuge tubes, weighed them to obtain wet mass and then dried them to constant mass within the open tubes in a desiccation chamber (a sealed plastic tub filled ¾ with anhydrous calcium sulfate, W.A. Hammond Drierite Company, Xenia, OH, USA). The wet and dry masses were collected for a separate experiment not reported here. We sealed the desiccated plugs within their original microcentrifuge tubes and stored them in a desiccation chamber for transport to and storage at the University of Sydney until DNA extraction. Blood samples and tail tips were taken the day after mating trials ended, so sperm and blood were sampled a maximum of 4 days apart. All animals were released at site of capture after two days of observation and appeared in good condition.

Quantifying relative telomere length

Sperm DNA was extracted following previously published protocols using a DNeasy Blood and Tissue Kit (Qiagen, Australia) with some minor modifications (Rollings et al. 2017a; b). The copulatory plugs were cut into smaller pieces and rehydrated overnight in 300 µL of deionised water at 36°C. Samples were gently centrifuged at 100 xg for 2 min and the water removed. DNA extraction then continued as previously published; the protein digestion step was allowed to run overnight to digest the proteins as fully as possible. DNA concentrations and the A260:A280 ratio, used to test for DNA purity, were measured with a Pherastar FS (BMG Labtech, Australia) and samples with ratio between 1.7-1.9 and a concentration above 10 ng/µL were included in the study without further processing. Due to the highly proteinaceous nature of the copulatory plugs protein

contamination was high. Where samples fell outside the range of 1.7-1.9 purification was attempted using sodium acetate and ethanol precipitation, statistical tests were performed to determine whether this additional purification affected the measured telomere lengths (see supplementary material for more information). If the purification did not produce an A260:A280 ratio within the acceptable range the samples were excluded from the study. DNA was successfully extracted from 38 copulatory plugs from 24 males. Due to multiple matings, 9 males contributed a total of 23 plugs to the overall sample size. Quantitative real time PCR was performed to measure telomere length relative to the reference gene *18S* according to previously published protocols, with blood and sperm samples from an individual placed in the same run (although the blood data was initially used for another study sperm and blood from any one individual were all assayed in a single run to minimise variation, Rollings et al., 2017a; b). Relative telomere lengths (RTL) were calculated by dividing the telomere signal by the *18S* signal generated for each individual and are thus unitless. See supplementary material for more information.

Ageing estimation by skeletochronology

Skeletochronology, which determines age to the nearest year, was performed according to previously published protocols using garter snakes (Rollings et al., 2017b) following methodology developed by Waye and Gregory (1999; 1998) and Clesson, et al. (2002). Skeletochronology is a useful method for estimating age in this species as the annular cycle of hibernation and reproduction means all offspring are born within approximately two months of each other, producing distinct, unambiguous cohorts each year just prior to overwinter dormancy. See supplementary material for more information.

Statistical analyses

All analyses were conducted with SAS 9.4 (SAS Institute, Cary, NC, USA). Sperm telomere length was ln transformed to improve adherence to normality according to the Shapiro-Wilk test (untransformed: p = 0.0101, transformed: p = 0.0742) and these transformed values used for all analyses. A one-way ANOVA was performed to test whether the additional purification step needed for some of the copulatory plug samples affected the measured telomere lengths. No significant difference was observed in STL ($F_{1,36}$ = 0.001, p = 0.976) or lnSTL ($F_{1,36}$ = 0.091, p = 0.764). A PROC MIXED analysis of the relationship between STL and copulatory plug number, with individual ID as a random factor, revealed no difference in STL between a male's successively inseminated ejaculates (n = 38, $F_{4,32}$ = 0.13, p = 0.9711). Therefore, we simplified the analysis, only the first copulatory plug from which DNA was successfully extracted was used in later analyses. This approach was chosen because males who mated multiply produced a smaller copulatory plug each time which presumably resulted in less sperm transference, less DNA, and likely decreases the probability of paternity (Friesen et al. 2013; Friesen, Squire, Mason 2014c). A correlation analysis between STL and ln transformed blood telomere length (BTL, as previously published in Rollings et al. 2017b) was performed, as well as a paired t-test to assess differences in telomere length. Multiple Pearson's correlation tests were conducted to compare STL, SVL, mass, and age. Both SVL and mass were correlated with age (r = 0.444, p = 0.0032, n = 42, and r = 0.361, p = 0.0188, n = 42, respectively) so a proxy measurement for growth rate was generated by calculating the residuals from a regression analysis of SVL against age and mass against age. A positive residual indicated individuals who were relatively longer or heavier than the mean of their cohort.

Results

Sperm and blood telomere comparison

Mean (± S.E.) relative STL and BTL of the subset of males for which sperm DNA was successfully extracted were 39.60 (± 2.81) and 24.66 (± 1.86), respectively (figure 1). STL was ~60.6 % greater than BTL (paired t-test, $t_{23} = -8.07$, $p < 0.0001$, $n = 24$). STL and BTL were positively correlated ($r = 0.641$, $p = 0.0007$, $n = 24$).

Sperm telomeres, size and age

Snout-vent length positively correlated with body mass ($r = 0.964$, $p < 0.0001$, $n = 42$) and age ($r = 0.444$, $p < 0.0032$, $n = 42$). Body mass positively correlated with age ($r = 0.361$, $p < 0.0188$, $n = 42$). STL increased with SVL ($r = 0.476$, $p = 0.0187$, $n = 24$; figure 2a) and approached significance with mass ($r = 0.401$, $p = 0.0521$, $n = 24$; figure 2b), but did not correlate with age ($r = 0.004$, $p = 0.9866$, $n = 24$; figure 2c). Thus, longer individuals had longer sperm telomeres.

Sperm telomeres and relative growth rates

STL was positively correlated with both the SVL and mass growth rate proxies which were generated from residuals of SVL and mass regressed against age (figure 3, $r = 0.524$, $p = 0.0086$, $n = 24$ and $r = 0.419$, $p = 0.0414$, $n = 24$, respectively), indicating that snakes with a relatively higher growth rate had longer telomeres. By contrast, BTL did not correlate with either the SVL or mass growth rate proxies ($r = 0.080$, $p = 0.6162$, $n = 42$ and $r = 0.023$, $p = 0.8828$, $n = 42$, respectively).

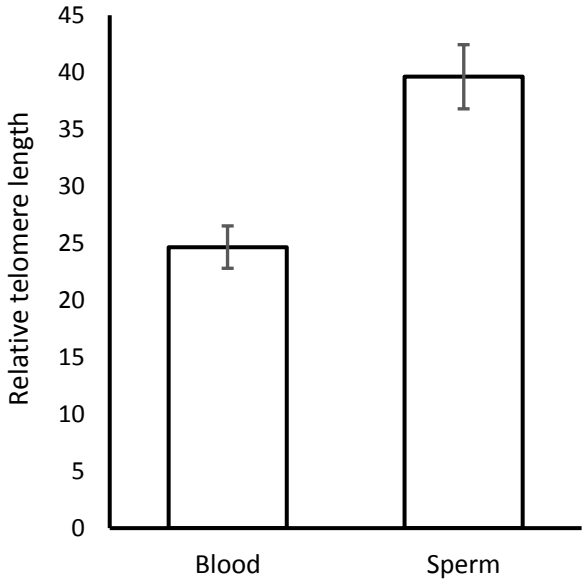

Figure 1: The mean (± S.E.) of relative telomere lengths of blood (BTL; n = 42) and sperm (STL; n = 24). STL was greater than BTL (paired t-test, df = 23, t = -8.07, $p < 0.0001$, n = 24). The data on blood is from previously published work (Rollings et al., 2017b).

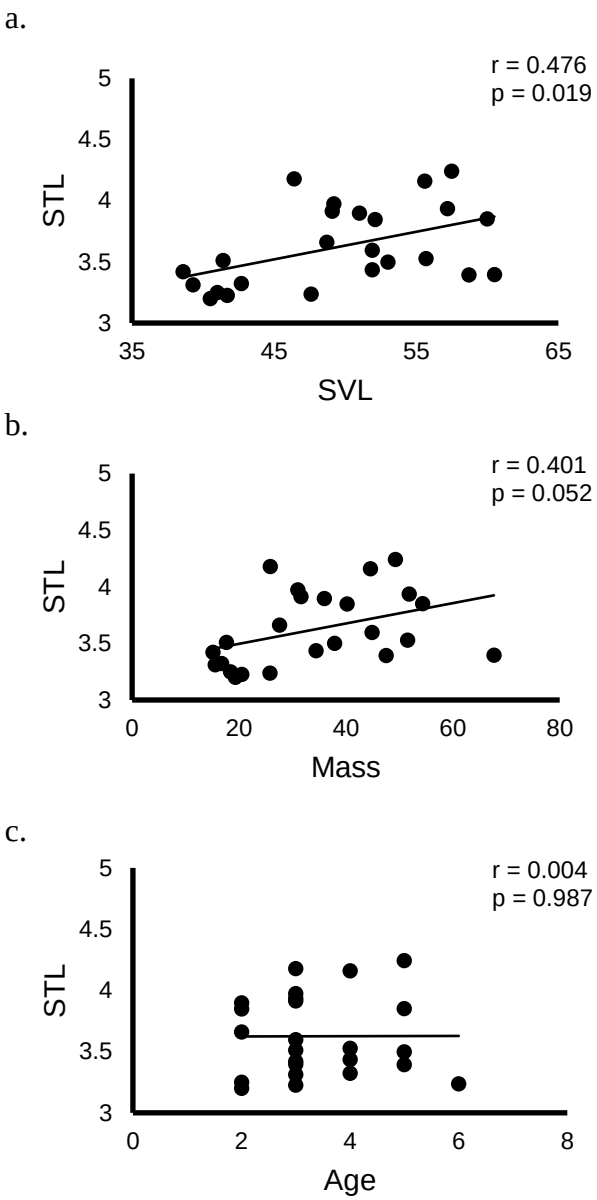

Figure 2: Correlations of ln-transformed relative sperm telomere lengths (STL) and physical traits. a: Snout-vent length (SVL, cm) is correlated with STL (n = 24). b: Mass (g) and STL approach significance (n = 24). c: Age (years) does not correlate with STL (n = 24).

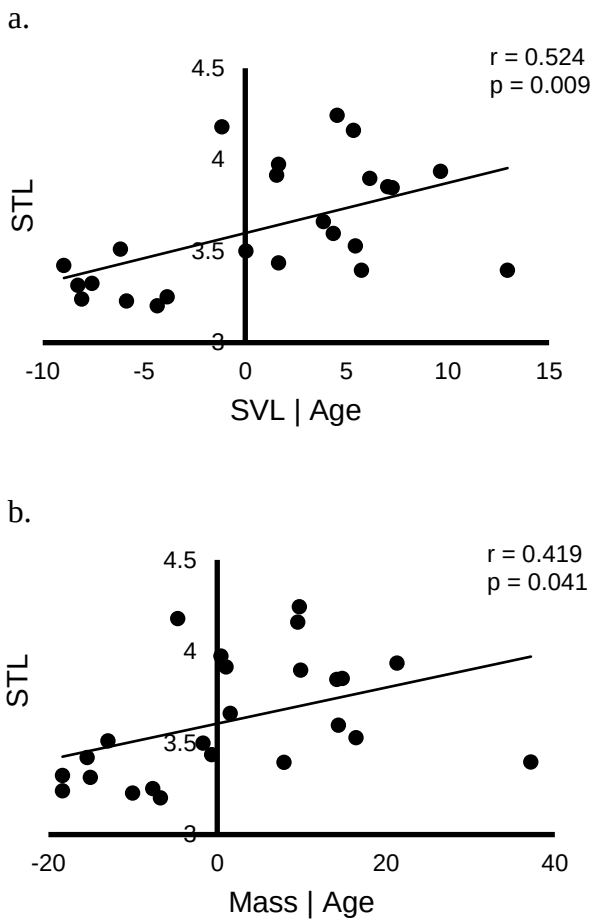

Figure 3: Correlations of ln-transformed sperm relative telomere lengths (STL) and the residuals of snout-vent length (SVL) and age and mass and age generated by regression analyses as proxies of relative growth rate. a: STL increases with SVL growth rate (n = 24). b: STL increases with mass growth rate (n = 24).

Discussion

Thus far, research on sperm telomere dynamics has primarily focussed on mammals, with a focus on humans, while little research has been conducted on other taxa. The present study, combined with our previous work (Rollings et al., 2017b), indicates differences in telomere dynamics between cell types in garter snakes. Male garter snakes have longer sperm telomeres than blood telomeres. This fits well with our understanding of the life history strategy of males in this species. Males greatly prioritise reproduction over growth

and cellular maintenance, compared with females. Blood telomere length declines with age in males while it remains stable in females (Rollings et al., 2017b); body condition (residuals from a regression of mass on length: (residuals from a regression of mass on length: Falk, Snow, Reed 2017)) also declines in males with age but is stable in female garter snakes. Males participate in every mating season for 2-4 weeks, but females only remain at emergence sites for 2-4 days before migrating to feeding grounds and frequently only become pregnant every second year (Gregory, 2009). As a trade-off of their high reproductive investment, males do not grow as large or live as long as females (Shine & Mason, 2005; Shine, Phillips, Waye, LeMaster, & Mason, 2003). While blood telomere length decreases with age in males (Rollings et al., 2017b), STL remains stable, suggesting the maintenance of STL is prioritised. Differences in telomere length may be explained by the different levels of telomerase activity between progenitor cell types (Blackburn, Epel, Lin 2015). Inherited telomere length may affect the life span of offspring (Noguera et al., 2018; Olsson et al., 2011), thus telomerase activity in the testes may maintain STL across life in order to maximise reproductive success. Due to the prioritisation of reproduction and because resources limited for fasting males at the same time they engage in intense mate searching and courtship, males may downregulate telomerase activity in haematopoietic cells, resulting in a gradual decline in blood cell telomere length with age in males, but not females. Comparisons of telomerase activity in the testes and haematopoietic cells of garter snakes need to be performed to determine whether that is the case. Regardless, the telomere dynamics of sperm and blood in male garter snakes diverge greatly and indicate the importance of cell- or tissue-specific telomere research.

Our results confirm our prediction that growth rate and STL are positively correlated. There are a few potential explanations for this relationship. Resource acquisition may affect growth rates and telomere length as resources are required for

growth and for the production of antioxidants and telomerase, which may preserve telomere length (Dmitriew, 2011; Dunham, 1978). Males are aphagic during not just hibernation but also the three weeks they remain at the den during mating season, reducing the amount of time available for foraging (Aleksiuk & Stewart, 1971). In addition, androgen concentrations may link growth rates and STL. The growth of male red-sided garter snakes is constrained by gonadal androgens, as has been observed in other reptile species with female-biased size dimorphism (Cox & John-Alder, 2005; Cox, Stenquist, & Calsbeek, 2009). Castrated garter snakes experience much higher growth rates which can be countered with testosterone implants (Crews et al. 1985; Lerner and Mason 2001). Androgens can reduce antioxidant defences, leading to high susceptibility to ROS damage which can cause telomere shortening (Alonso-Alvarez et al., 2007; von Zglinicki, 2002). Thus males who grow faster may have longer STLs because lower androgen concentrations result in less constraint on growth and mitigate telomere damage. However, we are restricted in our inferences as the study is cross-sectional and by the growth rate proxy we have used, which can only indicate relative growth rate, making us unable to assess how growth rates change with age. In order to determine the effects of growth rate in more detail, we would need to measure individuals repeatedly. Regardless, growth rate and STL are likely affected by both resources and androgens and further work is required to determine the relationships between these factors.

The extreme environment may also be strengthening the relationship between growth rate and STL, as both must occur within a limited timeframe. There are many subspecies of *Thamnophis sirtalis* and the majority of this species lives further south than the population of the present study, where such intense hibernation is not required (Fitch, 1981). Investigation of the sperm telomere dynamics of other subspecies may reveal

differences in growth and sperm production, potentially due to differences in life history strategy.

Determining the significance of STL also requires further research. We do not know whether STL is an overall indicator of sperm quality in garter snakes that could affect the probability of paternity. The production of high quality sperm may be particularly relevant to this species, as females mate multiply and can store sperm over the hibernation period for later use, which requires the sperm to remain viable over this timeframe (Friesen et al. 2014a). Comparison of STL with sperm quality metrics such as velocity and viability (Cariati et al., 2016) from freshly produced copulatory plugs and stored sperm may reveal whether STL is a predictor of sperm quality and longevity. In humans, sperm with longer telomeres are likely to be more successful in standardised swim-up trials, indicating a relationship between STL and sperm motility and viability (Santiso et al., 2010). A study in sand lizards, *Lacerta agilis*, observed that longer male blood telomeres result in a higher probability of paternity (Pauliny et al., 2018). While that study did not investigate STL specifically, in the present study the correlation between sperm and blood telomere lengths suggests that STL may be related to the probability of paternity in garter snakes. In order to determine whether STL relates to paternity and any transgenerational effects, we need to investigate the degree of heritability of STL and assess whether STL affects offspring viability.

Understanding whether blood telomeres correlate with other tissues is important as many studies on telomere length use blood cells due to the ease and relatively low invasiveness of collection (Barrett & Richardson, 2011; Olsson, Wapstra, & Friesen, 2018a). However, we do not have a good understanding of the relationship between blood cell telomere dynamics and the telomere dynamics of other tissue types. In the current study, we found a positive correlation between sperm and blood telomeres, but life history

traits interact with the telomeres of these cell types differently. It is possible that the correlation we have observed may be strengthened by interstitial telomeres, the quantity of which is currently unknown for red-sided garter snakes, which qPCR cannot exclude (Foote, Vleck, & Vleck, 2013; Olsson, Wapstra, & Friesen, 2018b), and thus this result requires confirmation with a method that can exclude interstitial telomeres, such as TRF. Regardless, our results suggest we cannot assume that the telomere dynamics of germline and somatic cells are similar and that life history strategies likely determine differences in dynamics.

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Supplementary full materials and methods

At the peak of breeding season (May 10, 2015), we collected snakes by hand from mating aggregations aiming to collect the full range of body lengths found at our Inwood, Manitoba study site (males: N = 100). We transported snakes to Chatfield research station, 16 km away, where they were weighed (±0.01g) and measured for snout-vent length (SVL: ±1mm). To ensure an equal distribution of sizes, we selected the 4 longest and 4 shortest individuals and an even distribution of intermediate sizes, obtaining a final sample of 42 males the remaining 58 animals were returned to the point of capture the next day. All animals were adults; juveniles are only rarely found at den sites (RTM > 25 years of pers. obs.; Gregory, 1977, Gregory, 1974). Blood (<0.1mL) for telomere analysis was taken from the caudal vein, added to 300 µL of RNAlater and frozen (-30˚C) until DNA extraction. Prior to collection of tails for skeletochronological aging, the tail of each animal was wiped clean with reptile ringers solution, and 95% ethanol, before receiving local anaesthesia. We injected 15-µL of 2% Lidocaine HCl subcutaneously and lateral to the vertebrae, 5 mm cranial to the site of tail excision. Ten minutes after Lidocaine injections, the distal 1-1.5cm of the tail was severed using a sterilized, single-edged razor blade on an aseptic field, and styptic powder was applied to the tail. (The 'surgical' field was cleaned with soap and water, then rinsed with 5% bleach solution between excisions. The razor was sterilized between uses: first cleaned with soap and water, rinsed with deionized water, then sterilized in a Germinator ® 500 dry sterilizer at 260ºC for >15 sec). The tail-tip was preserved in 10% formalin in neutral PBS, until histological analysis (see below). All animals were released at site of capture after two days of observation and appeared in good condition.

Skeletochronology/Histology

Individual age was estimated by a modified version of the technique described by Waye and Gregory (1998, 1999) and Clesson et al. (2002). Vertebrae were examined microscopically and the number of growth rings was identified for each animal. All animals were examined by two independent evaluators (HLW and RWK) in a blind test and the findings compared. Variation in age assessments (N = 3) were examined a second time by each evaluator and determined by consultation. This consultation consisted of RWK and HLW marking growth lines on photographs of the questionable sample sections to calibrate their readings, and then re-examining the outstanding slides to reach a consensus on a final age.

DNA purification

To purify contaminated DNA sodium acetate was added to the sample at a concentration of 0.3 M. 100% EtOH was added to increase the original DNA volume 3.5 times. The samples were then stored at -30°C for 40 minutes, before being centrifuged at 20 000 xg for 30 minutes at 4°C. The liquid was removed and 500 µL 70% EtOH added. The samples were gently shaken before being centrifuged at 20 000 xg of 15 minutes at 4°C. The liquid was removed and the samples allowed to air dry for 15 minutes at 37°C. The DNA was then resuspended in AE buffer (Qiagen, Australia).

Quantifying telomere length

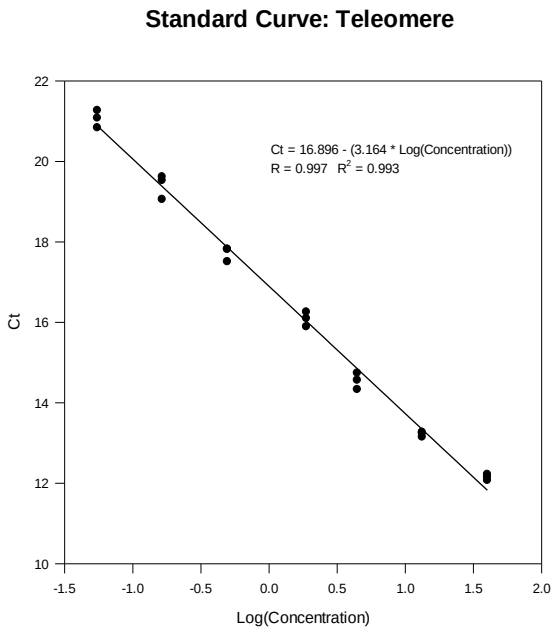
To analyse sperm telomere length we used a DNeasy Blood and Tissue Kit (Qiagen, Australia), and the DNA concentration (ng/µL) of each sample was measured in duplicate using a Pherastar FS (BMG, Labtech, Germany). Briefly, copulatory plugs were cut into smaller pieces and rehydrated overnight in 300 µL of deionised water at 36°C. Samples

were gently centrifuged and the water removed. DNA extraction then continued according to the manufacturer's instructions, the protein digestion step which was allowed to run overnight to completely digest the copulatory plug proteins. DNA was successfully extracted from the copulatory plugs of 24 males. Telomere length was measured using real-time quantitative PCR (qPCR) as we have done previously (Giraudeau et al., 2016, Rollings et al., 2017) using the 18S ribosomal RNA (18S) gene as the non-variable in copy number (NVC) reference gene. The melt curves produced for both telomere and 18S after amplification by qPCR displayed a single peak, indicating specific amplification of the DNA sequence.

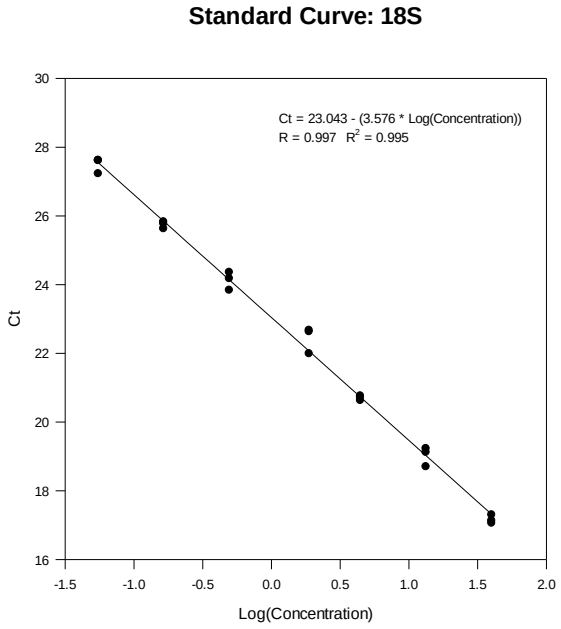
Reactions were run in triplicate for each sample. Amplifications were carried out in a Rotor-Gene 6000 thermocycler (Qiagen, Australia) using an initial Taq activation step at 95 °C for 10 min and a total of 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. A melt curve was generated after each run over the temperature range of 60 to 95 °C to ensure that there was no non-specific product amplification. Both the blood and sperm samples from an individual were included in the same run. No-template control reactions were run in triplicate for each primer set during every qPCR run to ensure that there was no contamination. Standard curves were produced, using the pooled DNA from four snakes, for both telomeres and 18S using three-fold serial dilutions to ensure consistent rates of amplification over a wide range of concentrations (40 ng/µL down to 0.055 ng/µL with 7 different concentrations in total: Supplemental Figure S1) giving a linear dynamic range of 0.055 to 40 ng/µL. The reaction was considered consistent the linear correlation coefficient exceeded 0.985. The efficiency of the telomere amplification was 1.01 and the efficiency of the 18S amplification was 0.90, and samples all fell within the same concentration range as our standards. All runs included the same 'golden standard' and also a no-template control to detect contamination. LinRegPCR 2016.0 (Ruijter et al.,

2009, Tuomi et al., 2010) was used to analyse the qPCR data. The starting concentrations of telomere (T) and control gene (S; 18S) as determined with LinRegPCR (below) were used to determine the relative telomere length with the calculation T/S. Telomeres and the control gene were assessed in separate runs. The mean inter-assay coefficient of variation for qPCR runs for telomere (n = 5) and 18S (n = 5) amplification were 11.6% and 18.0%, respectively, calculated using the golden standard. The intra-assay coefficient of variation for telomere and 18S runs was 15.1% and 14.4%, respectively. LinRegPCR generated efficiencies for telomere and 18S qPCR runs were 1.868 and 1.982, equivalent to 86.8% and 98.2%, respectively.

It is currently unknown whether the chromosomes of *T. sirtalis parietalis* contain interstitial telomeres (TTAGGG repeats found elsewhere in the chromosome; Foote et al., 2013) and, thus, whether they might influence the results. However, interstitial telomeres, which can vary in length between individuals, primarily add random noise to telomere measurements making the signal of telomere dynamics harder to detect (Foote et al., 2013, Smith et al., 2011).



Supplemental Figure 1: The telomere qPCR standard curve generated from a pooled DNA sample of four snakes. $R^2 = 0.993$ indicates the reaction proceeds linearly across the tested concentration range.



Supplemental Figure 2: The 18S qPCR standard curve generated from a pooled DNA sample of four snakes. $R^2 = 0.995$ indicates the reaction proceeds linearly across the tested concentration range.

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CHAPTER 7: General Discussion

My work, as shown in the previous chapters, indicates that telomere dynamics are highly dependent on life history strategies, showing a high degree of variability within species and even within individuals. In both painted dragons and red-sided garter snakes I have expanded our understanding of their life history strategies, helping to identify and explain trade-offs that are not immediately obvious.

In painted dragons I have found that red, blue, and bibbed males experience telomeric attrition in their blood cells across the mating season, whereas yellow males and those without bibs maintain their telomeric length (Chapter 4). Sperm telomere length also varies with morph, with orange and bibbed males having longer sperm telomeres than do the other morphs (Chapter 3). I also detected correlations in telomere length between six different cell types (blood, sperm, brain, liver, heart, spleen) and found that juvenile female dragons had longer telomeres than males across all of these cell types (Chapter 2), with the obvious exception of sperm. This work has expanded our understanding of trade-offs inherent in the processes that generate and maintain these different male morphs within the same population. Prior to my studies, red males were already known to dominate yellow males in physical contests, emerge from hibernation sooner, and have higher levels of testosterone (Healey et al., 2007, Olsson et al., 2007). By contrast, yellow males have larger testes and outcompete red males in sperm competition, particularly where sperm storage has occurred (Olsson et al., 2009b). My research has further revealed an additional trade-off associated with the “red male” strategy as this morph has much higher telomeric attrition than do yellow males. Interestingly, no significant difference in sperm telomere length was observed between red and yellow males, suggesting that telomere length may not be a factor in the superior sperm competition ability of yellow males.

Bibbed males lose body condition more easily during the mating season than do males without bibs, but are more effective at maintaining exclusive paternity of clutches (Olsson et al., 2009a). That pattern led Olsson et al. (2009a) to speculate that bibbed males may invest more effort into reproductive behaviour than do non-bibbed males. My analysis of the telomere dynamics of bibbed males is consistent with this hypothesis. Bibbed males experienced higher blood cell telomeric attrition across the mating season than did males without bibs, suggesting they do invest less into cellular maintenance than do non-bibbed males. Telomeric attrition may also entail a trade-off, as production of the bib is likely energetically expensive. By contrast, bibbed males had longer sperm telomeres than did males without bibs. Sperm telomere length may not necessarily influence sperm competitive ability, as no significant difference in sperm telomere length was observed between red and yellow males. Nonetheless, sperm telomere length may indicate overall investment into sperm production and maintenance.

My investigation of telomere lengths in juvenile dragons clarifies the results of previous research on the eggs of painted dragons. Testosterone levels differed significantly between eggs that gave rise to male versus female dragons (Tobler et al., 2011). Exposure to heightened testosterone may accelerate sexual development in the males, but those high testosterone levels may also act as an oxidant (Alonso-Alvarez et al., 2007), accelerating telomeric attrition. I observed that juvenile male dragons had shorter telomeres than did juvenile females, across a wide variety of cell types. As the majority of dragons experience only a single mating season there is evolutionary pressure to mature rapidly; and as a result, telomeric attrition and accelerated senescence may be a small penalty for successful reproduction. In summary, my research on painted dragon telomeres has built upon a well-established body of knowledge, allowing further characterisation of the life history

strategies of the species and exploring some of the ways in which telomere dynamics and trade-offs interact.

My research on red-sided garter snakes has furthered our understanding of differences in life history strategy between males and females. Male garter snakes have shorter telomeres than do females, and experience a decline in blood telomere length and body condition with age. In contrast, females maintain their telomeres and body condition (Chapter 5). Telomere lengths in sperm and blood were correlated in male garter snakes, and sperm telomeres were significantly longer than those in blood. Sperm telomere length also was correlated with snout-vent length and relative growth rate (Chapter 6). The life history and prioritisation of reproduction by male garter snakes has already been characterised in detail (see Shine, 2012 for a synthesis of the strategies of the species) but my research further clarifies the costs and benefits of such a strategy. Previous research observed a decline in body condition in males across the mating season due to aphagy (Shine & Mason, 2005, O'Donnell et al., 2004). However, my research has also shown a loss of body condition in males with age, indicating that despite spending the summer months feeding they are not able to fully compensate for the energetic demands of their reproductive strategy. The cost of such a strategy is further reflected in the telomeric attrition experienced by males, especially when compared with the stability of telomere length and body condition in females. The decline in body condition and likely accelerated senescence in the males may also explain why the females captured for the study in Chapter 5 were significantly older (on average) than the males. I observed that while blood telomeres shorten with age in males, sperm telomeres remain stable. Sperm telomere lengths likely remain stable due to higher telomerase or antioxidant activity, or a combination of both, within the testes. That investment would require yet more resources for reproduction. My research has thus identified another aspect of the extreme

reproductive tactics of males in this extreme-climate population. The correlation between sperm telomere length and relative growth rates suggests an underlying biological mechanism that may constrain both. Growth and sperm telomere length may be limited by resource availability, or androgen concentrations (which constrain growth in garter snakes and may act as oxidants; Cox & John-Alder, 2005, Cox et al., 2009, Crews et al., 1985, Alonso-Alvarez et al., 2007). Thus, androgen levels may affect growth and telomeric attrition simultaneously. My studies of the telomeres of red-sided garter snakes have revealed further differences between the sexes, caused by their greatly divergent life history strategies.

By investigating two polymorphic species with distinct life history strategies I have identified similar trends in telomere dynamics and life history strategies in both species. Telomere stability was observed in individuals that maintain body condition, such as male yellow painted dragons and female red-sided garter snakes. Conversely, male red painted dragons and male red-sided garter snakes trade-off body condition for aggression and reproduction, respectively, and experience higher telomeric attrition. Males with bibs, which may be used to attract females (McDiarmid et al., 2017), also experience greater telomere loss and lose body condition more easily than do non-bibbed males (Olsson et al., 2009a). Both female adult garter snakes and juvenile painted dragons had longer telomeres, on average, than their male counterparts. In both species, males had longer sperm telomeres than blood telomeres.

Although some of my results are not directly comparable between the two species as they were not studied in identical ways, the data may still provide insight into relationships between life history strategies and telomere dynamics. For example, female garter snakes maintain blood telomere length and body condition across their life span, whereas males experience a decline in both. Juvenile male painted dragons have much

shorter brain telomeres than do juvenile females. Although the reason for this is unknown, one hypothesis is that males experience rapid brain development, required to effectively defend territories and females. Sperm telomeres are longer in orange males and those with bibs, suggesting that increasing the likelihood of offspring survival may be central to their life history strategy. Alternatively the longer sperm telomeres of the orange males may be a consequence of an upregulation of antioxidants in the testes to produce higher quality sperm with less oxidative damage.

More life history research is needed to test these hypotheses. Previous research on telomere length heritability in painted dragons has focussed on mothers (Ballen et al., 2012). Assessment of sire morph-type and sperm telomere length along with offspring survivorship is required to determine whether the manipulation of sperm telomere length is a strategy used within this species or whether it is largely a function of cellular division and telomerase activity within the testes. To test whether male dragons do experience rapid brain development, relative to females, a comparison of cognition in juvenile and adult dragons of both sexes is required. Cognition is also likely to vary among individuals, regardless of sex, and so an overall relationship between cognitive ability and brain telomere length should also be researched. Differences in cognitive development and brain telomeres may also be assessed by raising dragons individually or in groups, as the cognitive ability of interest requires the ability to interpret the behaviours of other dragons. Brain telomere length may also be investigated in the garter snakes. Although male and female garter snakes behave differently during the mating season, many individuals experience multiple mating seasons and males do not patrol territories. As such, rapid brain development may not be under selection in this species. We may predict then that garter snakes would be unlikely to have such a pronounced difference in brain telomere length as compared with the dragons. While my research on these two species has revealed multiple

trends between telomere dynamics and life history strategies, much more remains to be investigated.

Research on telomere dynamics also needs to focus on a broader range of taxa. Most research has involved endotherms (Olsson et al., 2018), and frequently those with low levels of sexual dimorphism, and thus low levels of sex-specific variation in life history strategies. In many species investigated thus far, no significant difference in telomere length between the sexes has been observed (Barrett & Richardson, 2011), in contrast to my observation of sex-based differences in telomere dynamics in both garter snakes and painted dragons. In order to test the relationship between life history strategy and telomere dynamics, a much greater variety of species with unusual life history strategies needs to be assessed. For example, male side-blotched lizards, *Uta stansburiana*, as discussed in the introduction of Chapter 4, have a complex reproductive hierarchy where the three colour morphs have a "rock-paper-scissors" system whereby one morph reproductively dominates another while losing to a third (Sinervo & Lively, 1996). If the relationship between life history strategy and telomere dynamics observed in the painted dragons is consistent across species we would predict that the aggressive orange-throated morph in the side-blotched lizards would have shorter telomeres than the yellow- and blue-throated morphs. The females of this species also have two colour morphs, orange and yellow, with orange being more aggressive and producing larger clutches of smaller eggs than yellow females (Alonzo & Sinervo, 2001). Orange females may therefore have shorter telomeres than the more gregarious yellow females. The offspring of orange mothers may also have shorter telomeres as the resources used by the mother in reproduction must then be spread across a larger number of individuals. Alternatively, there may be no difference in telomere length in the offspring of orange mothers if telomerase is upregulated to compensate. However, as the upregulation of telomerase would require additional

resources this may result in a lower body condition in orange mothers. Assessment of the telomere dynamics of the different morphs would allow us to test how consistently aggression relates to telomeric attrition and provide an opportunity to analyse the telomere dynamics of females with morph specific behaviours.

Many types of intraspecific polymorphism exist across the animal kingdom and they provide a great opportunity to further our understanding of telomere dynamics while minimising genetic variation that may obscure trends. To know whether the relationships between telomere dynamics and life history strategies I have observed are consistent we need to study more than just vertebrates. While telomere dynamics may vary greatly between species, telomere structure is fairly consistent throughout Metazoa (Gomes et al., 2010, Traut et al., 2007). As such, any polymorphic species with unusual life history strategies may help advance our understanding of telomere dynamics. Sexual dimorphism, in the form of displays by males to attract females, are common in insects. Nuptial gifts, edible objects given by a male to a female prior to copulation (Gwynne, 2008), presumably involve a trade-off for the male as resources gathered by the male are then transferred to the female. This may result in a shortening of his telomeres, but also potentially increase the resources his offspring receive if he mates successfully. In a species of damselfly, *Ischnura elegans*, females are colour-polymorphic either appearing as a brown colour, similar to the males, or a bright blue, unique to the females (Sánchez-Guillén et al., 2013). Male preference for these morphs depends on the morph frequency, but males typically need to learn to recognise the blue females as a viable mating option. This is advantageous for blue females as repeated mating typically produces diminishing returns for female damselflies (Sánchez-Guillén et al., 2013). However, the evolutionary pressure to produce their bright blue colour would likely make them greater targets for predation and require energy to produce, potentially reducing body condition. As such, we may predict blue

damselfly females would have shorter telomeres than their brown conspecifics and brown females in particular. Polymorphic species provide a variety of opportunities to test hypotheses of telomere dynamics.

However, the future directions I have suggested thus far are largely observational or correlational in nature. To advance our understanding of telomere dynamics a reliable, and non species-specific, method of telomere manipulation must be developed. It is currently unknown whether telomeric attrition affects lifespan or whether it is simply a measure of other biological processes. At present research on telomere dynamics typically relies on observation or manipulation of an external factor, such as temperature or diet, to assess whether these factors affect telomeric attrition. However, in order to determine whether telomere dynamics affect an organism directly we must be able to manipulate telomere length. The difficulty in developing such a technique lies in the fact that telomere length is so tightly interlinked with many other cellular processes. Any methodology developed must only affect telomere length so as not to confound results. For example, while using L-buthionine sulfoximine to reduce antioxidant production shortens telomeres, it also damages other macromolecules (Cattan et al., 2008), likely affecting the overall health of the organism. Manipulations of ROS or antioxidants may be effective at answering ecologically-based questions but cannot determine whether telomere dynamics have a causal effect on ageing and lifespan. Some experimental manipulation of telomere length has occurred through the generation of mice and zebrafish with inactive telomerase (Henriques et al., 2013, Sacco et al., 2010, Rudolph et al., 1999, Wong et al., 2000), with reduced lifespan and cellular degeneration reported. When telomerase is reactivated in mice, telomerase-deficient cell proliferation increases and organ function improves (Jaskelioff et al., 2011). While these results are interesting they are restricted to just a couple of species and the methodology cannot be applied to wild populations. Although it

was unsuccessful, in theory the attempt at telomere manipulation via oral administration I report in Chapter 4 presents a simpler approach that may be more broadly applicable to the research of telomere dynamics. The compound tested, TA-65 (cycloastragenol), was selected as it was previously observed to increase telomere length in zebra finches and increase feather regeneration rate (Reichert et al., 2014). However, the metabolic systems of endotherms and ectotherms differ greatly and this may affect how the compound is processed in birds compared with reptiles. Further investigation of the compound used, and others like it, is needed to assess whether the null result in Chapter 4 was due to methodology or the compound itself.

Telomere dynamics are affected by a confluence of many biological factors: organismal growth, molecular damage, cellular maintenance, and reproduction all affect telomere length. The potential consequences of telomeric attrition or lengthening are not consistent across species. However, the challenges of understanding causal factors for variation in telomere dynamics may be slightly reduced when those dynamics are considered from the perspective of life history strategies. By studying polymorphic species with distinct strategies we may not only quantify extensive variation in telomere dynamics, but also we will be able to compare those variants more easily due to lower divergence between morphs in genetic traits (relative to interspecific comparisons). Polymorphic species are an invaluable evolutionary asset that can help us determine the relationship between life history and telomere dynamics.

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