2018

The telomere hypothesis of stress and aging

Michael Todd Shultz
Iowa State University

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by

Michael T. Shultz

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Ecology and Evolutionary Biology

Program of Study Committee:
Anne M. Bronikowski, Major Professor
Stephen Dinsmore
Eric Henderson

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

DEDICATION

This thesis is dedicated to Jesse Sykes—if I had not wanted to impress you so much, I would not have worked so damn hard and this dissertation would not be nearly as interesting; to Chris Hovnanian—an astounding intellect and amazing friend—I am eternally grateful that you were able to find the strength to stick around long enough to see me through this; to my parents for their unwavering support and patience; and to everyone who is awestruck by nature yet courageous enough to not bow at its altar. I have expended a tremendous amount of energy in this process, but you had the toughest job. This belongs to you.

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ACKNOWLEDGEMENTS

I would like to thank faculty of Iowa State University who invested their time and energy to help me succeed in achieving my goals—in particular, I wish to thank Dr. W. Graves, Dr. K. de Laplante, Dr. S. Dinsmore, Dr. Eric Henderson, and Dr. J. Wendel for their continual support and guidance.

In addition, I would also like to thank my friends and fellow graduate students—especially Dr. D. Green and Dr. T. Mitchell.

Finally, thanks to my family for their encouragement, C. Hovnanian for his comradery and intellectual contributions, and to Jesse Sykes for her hours of patience, respect and love

CHAPTER 1. INTRODUCTION

Telomeres are repetitive sequences of DNA forming the ends of chromosomes in eukaryotic organisms and are the focus of human aging studies. Cells accumulate wear and tear from day to day use, so they replicate, but their DNA can accumulate damage over time and be multiplied with each cell replication with harmful effects on the organism. Telomeres are thought to limit the lifespan of cells by 'counting' the number of cell replication events and triggering cell senescence or death when the maximum number of safe cell replications has been reached. Over time, these senescent cells accumulate and produce the physical signs of aging. However, tests of this hypothesis in non-model organisms do not support predictions suggesting the predominant theory of telomeres and aging is incomplete.

In chapter 1, I propose an alternative hypothesis of telomere function and its relationship to stress and aging called, "The Telomere Hypothesis of Stress and Aging" (THSA) which incorporates an additional, previously underappreciated telomere function—their role in modulating expression of stress response genes. Genomic DNA is compacted into chromatin—a complex folding architecture which alters the activity of specific genes. Environmental stress triggers telomere shortening, cell metabolism slows, chromatin unfolds, and stress response genes are upregulated. Following chromatin remodeling some telomeres are re-lengthened and cell metabolism increases. Thus, the THSA predicts dynamic telomere length and cell metabolic rate that are related to levels of environmental stress exposure. In addition to formalizing the Telomere Hypothesis of Stress and Aging (THSA),

If birds have higher levels of ALT than mammals, they should also have a greater capacity to re-lengthen telomeres in general. Thus, according to predictions of the THSA, on a gram for gram basis, birds are expected to lose telomeres more slowly with age than mammals. Whether or not this is true was previously unknown. I examine this question in Chapter 2; by comparing the rates of telomere loss with age between birds and mammal species. When comparing rates of telomere loss with age between birds and mammals, results were as predicted—for a given body size, birds lost telomeres with age at slower rates than mammals. Thus, results support the THSA—if birds possess higher rates of ALT this may account for their ability to achieve longer lifespans for the body size and metabolic rate. Longer-lived species of birds and mammals lost telomeres with age at slower rates than short lived ones. This is consistent with both the predominant paradigm and the THSA, except the THSA predicts a portion of telomere shortening with age is due to increasing up-regulation of stress response genes with age.

CHAPTER 2. THE TELOMERE HYPOTHESIS OF STRESS AND AGING

Abstract

Studies of telomere length, age, and fitness relationships, particularly ecological ones, have tested predictions of the Telomere Hypothesis of Cellular Aging (THCA), but individual variation in telomere length within age-classes is larger than predicted and the THCA does not provide an alternative hypothesis. Here, I propose an alternative hypothesis "The Telomere Hypothesis of Stress and Aging" (THSA) accounting for telomere regulation of gene expression in response to environmental stress. When exposed to environmental stressors, telomere truncation facilitates chromatin remodeling and changes in gene transcription promoting stress resilience. Chromatin remodeling is determined by epigenetic modifications to telomeric histone proteins and sub-telomeric DNA, providing selective access of transcription factors to DNA. The length of telomeres and their folding structure permits their direct interaction with genomic DNA to alter gene expression. A form of normal telomere recombination (ALTn), working in a coordinated manner with telomere transcribing RNA (TERRA), modulates telomere length in response to environmental stress in a diverse range of taxa, including humans. The capacity of an individual to use telomerase to alter telomere length (TERT telotype) versus ALTn, results in three telotypes (ALT1, ALT2, and TERT). These telomere types differ in length and can differ in ratio of occurrence relative to stress severity. Predisposition for a principle telotype, or stress resilience, is inherited non-genetically,

and may penetrate multiple generations depending on reinforcement in subsequent generations. This establishes a strong predisposition for the telotype ratio, but it can be modified in response to early life stress (ELS), and to lesser extent, throughout life depending on subsequent stress exposure.

The "Telomere Hypothesis of Stress and Aging"

The Telomere Hypothesis of Stress and Aging (THSA) proposed here is distinct from the Telomere Hypothesis of Cellular Aging as it includes observations of telomere length changes associated with expression of genetic pathways promoting stress resilience, in addition to telomere shortening owing to the end-replication problem.

The theory makes several unique predictions—

1) The existence of three telomere states or 'telotypes' with different TL set points, are directly related to their function in gene expression related genetic pathways conferring stress resilience (ALTI, ALTII, TERT).

2) All three telotypes should occur simultaneously within individuals

3) The telotypes should differ in their replication and metabolic rates.

4) Telomere length is expected to vary among individuals of the same age significantly more than predicted under the THCA and complementary theories (metabolic theory of aging and oxidative stress hypotheses).

5) The ratio of telotypes reflects the 'anticipated' need for stress resilience, which is determined by environmental stress exposure frequency and intensity.

6) A predisposition for stress resilience, and thus an individual's TL is non-genetically inherited, with strength of the predisposition varying relative toto; a) previous generations' stress exposure, b) maternal stress exposure, and c) early life stress (ELS).

7) Lastly, the ratio of telotypes is predicted to confer plasticity in gene expression with pleiotropic effects that regulate life-history traits, and is therefore predicted to vary among individuals, species, and taxon accordingly.

Telomeres and Aging—"The Telomere Hypothesis of Cellular Aging"

Telomeres are concatemers of non-coding DNA located at the termini of linear chromosomes and are generally 5-8 base pairs in length (E. H. Blackburn and Gall 1978). In vertebrates, telomeres are six base pairs (TTAGGG) (MEYNE, RATLIFF, and MOYZIS 1989), repeated thousands or tens thousands of times depending on the species and tissue type (E.H. Blackburn and Szostak 1984). Telomeres function to maintain chromosome stability (McClintock 1934; Muller 1938) and proper DNA replication (A.M. Olovnikov 1973; Alexey M. Olovnikov 1996). Telomeres shorten with each round

of cell replication until cells must cease replicating to prevent the proliferation of inaccurate DNA or chromosome aberrations such as end-end fusions (Counter et al. 1992). The proportion of senescent and dead cells increases over time and are believed to be responsible for the phenotypic traits of organismal aging. Thus, telomeres are the focal point for studies of human aging and age-related diseases, as evident from an ISI Web of Science search of "telomere" yielding more than 50,000 studies published to date, which equates to greater than 5000 per year, and >50 per day.

The Telomere Hypothesis of Cellular Aging (THCA) forms the theoretical foundations of nearly all ecological studies of telomeres (Harley et al. 1992) and makes two fundamental assumptions about telomere length; (1) a small amount of DNA from telomeres is lost with each round of DNA replication (i.e., 'principle of marginomity', (Olovnikov 1971) 'the end-replication problem'; Levy et al. 1992), and (2) these deletions accumulate, limiting the number of cell replications and leading to cell senescence or programmed cell death (i.e., apoptosis) (Harley et al. 1992). Thus, implicit to the theory is that on average telomere length is positively related to cell and therefore organismal viability.

The THCA also makes several assumptions about telomere extension —1) telomere length can be partially restored by the action of telomerase—a ribonucleoprotein reverse transcriptase composed of a catalytic reverse transcriptase subunit (TERT) and RNA subunit (TER) (Greider and Blackburn 1985; Greider and Blackburn 1987); 2) however, telomerase activity is largely confined to tissues and cells requiring high rates of proliferation— e.g., stem cells, germ cells, embryonic tissues, and

is suppressed in somatic cells, such that telomerase does not replace all lost telomere sequences; 3) telomerase is rarely reactivated in somatic tissues and when it is reactivated, it represents a dysfunctional state, cell crisis, or oncogenesis (Harley and Villeponteau 1995; Jennifer Prescott et al. 2012), and 4) telomeres are occasionally repaired by homologous recombination (HR, or the exchange of telomere sequences between homologous telomere strands), but this is also dysfunctional and indicative of cell crisis (Harley et al. 1992).

It is generally understood that oxidative stress also contributes to telomere shortening (von Zglinicki 2002), although it is not formally included in the THCA. The extent of telomere shortening due to oxidative damage is inversely related to the level of cell antioxidants (Xu, Neville, and Finkel 2000) and positively related to the generation of reactive oxygen species (ROS) during cellular respiration (Caldini et al. 1998). In sheep and humans, *in vitro* oxidative stress is associated with ~25% more telomere shortening than expected from replicative loss (Richter and von Zglinicki 2007).

Telomeres in Ecology and Evolutionary Biology

Ecologists were introduced to telomeres in 2002, when the first of a series of seminal papers were published testing foundational predictions of the THCA in non-model organisms— telomere length (TL) declined predictably with age in Zebra Finches *(Taeniopygia guttata)* (M. Haussmann and Vleck 2002), long-lived bird and mammal species lost telomeres with increasing age at a slower rate than short-lived species (M. F. Haussmann et al. 2003), and telomerase activity was higher for long-lived than short-

lived bird species (M. F. Haussmann et al. 2004). Collectively, the evidence suggested it might be possible to calibrate a species' mitotic clock (Mark F. Haussmann, Vleck, and Nisbet 2003) to estimate an individual's chronological age and relative rate of aging with a single, non-destructive tissue sample. Much excitement ensued and since then, more than 250 ecological studies of telomeres have been published (according to a search of "telomere and ecology" in ISI's Web of Science).

However, as data has accumulated, enthusiasm has been tempered because of the consistent observation that chronological age accounts for little variation in telomere length (<15% of telomere length) such that the range of telomere length for the youngest and oldest age classes of a population overlap (e.g., (M. F. Haussmann et al. 2003). This is much more variation in telomere length than predicted by the THCA and has led to a number of reviews on the topic. Conclusions and recommendations made by these authors fall into several categories; 1) telomere length data are accurate and precise, and future effort should focus on understanding the biological sources of the variation, 2) telomere length data are inaccurate and/or imprecise owing to technical challenges of laboratory and/or analysis methods, and 3) inconsistencies in laboratory and analysis methods make interpretation difficult and methods should be standardized (e.g., Pat Monaghan 2010; P. Monaghan et al. 2008; Ricklefs 2008; Aviv 2008; Nussey et al. 2014). However, evidence suggests a more parsimonious explanation—the THCA is simply incomplete.

Since the THCA was formalized, it has become increasingly evident that the utility of the theoretical framework is limited because it does not include a critical

function of telomeres—that they regulate the transcription of pleiotropic genes, which collectively function to optimize resource allocation in response to environmental challenges, or more concisely—stress resilience. The remainder of this chapter reviews evidence that telomere-mediated changes in gene transcription have been demonstrated to affect cellular and organismal metabolic rate, proliferation, growth rate, lifespan, and life-history trade-offs, with gene expression requiring changes in telomere length. Therefore, relationships between telomere length and organismal or cellular viability are non-linear and possibly bimodal. (Feel like you need refs for the above statements or at least a statement saying you will review evidence below).

Telomere Regulation of Gene Expression

Telomeres and sub-telomeres (the region just below telomeres) consist of facultative heterochromatin and have a dynamic, complex, folding structure, or conformation, functioning to alter gene expression (Galati, Micheli, and Cacchione 2013). Although telomeres consist of non-coding DNA, they alter gene transcription by binding with transcription factors and physically interacting with genomic DNA. In humans, the average length of telomeres is about 9Kb, but they can alter gene expression at least 10 Mb distal to the telomeres (Baur 2001; Tennen et al. 2011; Robin et al. 2014). The majority of the effected genes are located in the sub-telomere and these have pleiotropic, cascading effects related to stress resilience (Robin et al. 2014; Pedram et al. 2006; Tennen et al. 2011).

Telomere and signal protein interactions are mediated by epigenetic modifications to telomere-associated proteins and sub-telomeric DNA; including acetylation, methylation, phosphorylation and ubiquitylation. Telomeres are packaged in nucleosomes—telomeric DNA is wrapped around an octamer of histone proteins (four proteins, each occurring twice). Epigenetic factors covalently bind amino acid residues of histone protein 'tails', relaxing their ionic bonds with DNA. This 'opens' DNA to selectively attract gene-expression enhancing and repressing protein factors (Doheny, Mottus, and Grigliatti 2008; Ettig et al. 2011; Episkopou et al. 2014).

Histone acetylation is an important epigenetic modification of telomere and sub-telomere chromatin, which primarily enhances gene expression (Jacobs 2002; Michishita et al. 2009; Michishita et al. 2008; Jeon, Yoo, and Chung 2010; Tennen and Chua 2011; Kanfi et al. 2012). Conversely, de-acetylation of histones limits protein access to DNA, repressing gene expression (Galati, Micheli, and Cacchione 2013; Blasco 2007; Gottschling et al. 1990).

Histone methylation is an epigenetic regulator of telomere chromatin state that forms evolutionarily conserved 'epi-alleles', (e.g., methylation of histone 3 (H3K79) occurs in *Drosophila* (Perrini et al. 2004), budding yeast *Saccharomyces cerevisiae* (Nimmo, Cranston, and Allshire 1994), mice (A. T. Nguyen and Zhang 2011) and humans (Arnoult, Van Beneden, and Decottignies 2012). Histone methylation has specific and selective effects on gene expression—for example, in human leukocytes histone methylation is positively related to the activity of 47 promoters and negatively

related to the activity of 27 promoters mostly located in the sub-telomere (Buxton et al. 2014).

DNA methylation requires CpG sites, so mammalian telomeric DNA cannot be methylated (as it contains only TpT, TpA, GpG), but sub-telomeric DNA contains CpG sites and is often heavily methylated (Vera et al. 2008; Galati, Micheli, and Cacchione 2013). Sub-telomere methylation increases heterochromatin formation, which can eventually spread to telomeres to reinforce repressive effects on gene expression (Gonzalo et al. 2006; Pedram et al. 2006). DNA and histone methylation are at times independent of one another, but histone methylation can promote the recruitment of methyl groups to sub-telomeric DNA (Cedar and Bergman 2009). Thus, collectively evidence indicates telomere conformation, or chromatin state, is affected by epigenetic factors and telomere chromatin state ('open' or 'closed') alters gene expression. Telomere chromatin state and its role in regulating gene expression is not included in the THCA, but alters predictions related to telomere length, environmental and organismal stress, as well as aging.

Telomere Length and Gene Expression are Dependent on Chromatin State

Epigenetic modifications that 'remodel' telomere chromatin are also associated with telomere length changes—telomere truncation events result in increased access to DNA by transcription factors (Benetti, García-Cao, and Blasco 2007). The opposite is also true—long telomeres are more heterochromatic with more 'closed' chromatin, which is generally more repressive to gene expression (Benetti, García-Cao, and Blasco 2007).

Thus, telomere length is directly related to changes in gene expression and these are associated with epigenetic modifications to telomeric DNA.

Histone methylation often has “On” or “Off” effects on gene expression. For example, in *S. cerevisiae*, sub-telomere histone methylation (H3K79) de-acetylates H4K16, truncating telomeres, and turns gene expression “ON” (Kitada et al. 2012). In mice, increased sub-telomere methylation is associated with long telomeres and repressed expression of the sub-telomeric trans-neomycin gene (Weuts et al. 2012). In humans, over-expression of hTERT (telomerase reverse transcriptase) lengthens telomeres, increases heterochromatin compaction, and represses sub-telomere transgene expression (Baur 2001). The process is reversed by overexpression of TRF1 (a binding protein that inhibits the ability of telomerase to extend telomeres), truncating telomeres and enhancing sub-telomeric transgene expression (Koering et al. 2002). In humans, sub-telomere DNA methylation is positively related to telomere length, heterochromatin compaction, and repressed transcription (Arnoult, Van Beneden, and Decottignies 2012; Cusanelli, Romero, and Chartrand 2013, Buxton et al. 2014). The effect decreases with age—adolescents have greater sub-telomere methylation, longer telomeres, more heterochromatin compaction, and repressed sub-telomeric gene expression compared to adults (Hewitt et al. 2012, Maeda et al. 2009).

Telomere length-mediated gene expression patterns are often referred to as “the telomere position effect” (TPE), reflecting that genes closer to telomeres are more likely to be silenced. However, the term is misleading because TPE ‘selectively’ silences genes, often long distances from telomeres (Vega-Palas, Martin-Figueroa, and Florencio 2000;

Fourel et al. 1999). In *D. melanogaster* at least 30 loci throughout the genome are regulated by TPE with many more remaining to be identified (Doheny, Mottus, and Grigliatti 2008). In mice, TPE silences genes of the inactive X chromosome (Schoeftner and Blasco 2008). In humans, 'telomere position effect over long distances' or TPE-OLD can have both repressive and enhancing effects on genes as far as 10 Mb distal from telomeres (Robin et al. 2014). The selectiveness of TPE is mediated by chromatin remodeling transcription factors; for example, in *S. pombe*, Abf1p binds telomeric DNA, reduces telomere heterochromatin compaction, and eliminates TPE (Fourel et al. 1999; Miyake et al. 2004).

Telomere Recombination (Normal ALT) Facilitates Length Dynamics

A form of homologous recombination (HR), likely associated with telomere transcribing RNAs (TERRA) modulates telomere length with subsequent effects on gene expression effects and increased stress resilience. Although, the THCA assumes HR is dysfunctional (Harley et al. 1992), it is the sole mechanism available to restore lost telomere sequences for a number of organisms (e.g., fruit fly *Drosophila spp*.(Harald Biessmann et al. 2000)onion *Allum cepa,* (Pich and Schubert 1998); the mosquito *Anopheles gambiae* (H. Biessmann, Donath, and Walter 1996), the midge *Chironomus* (Nielsen and Edström 1993); and may be the ancestral mechanism of telomere length regulation, in general (Louis 2002; Fajkus, Sykorova, and Leitch 2005; Gomes et al. 2011). Telomere length maintenance using telomerase during development, followed by telomerase suppression may have evolved in response to increase mutation risk

associated with the evolution of homeothermy in mammals (Gomes et al. 2011). This theory is supported in the case of the naked mole rat *(Heterocephalus glaber)*, a species known for its exceptional longevity, as they appear to utilize HR to regulate TL (Kim et al. 2011) and have a limited capacity for thermoregulation relative to most mammals (Goldman et al. 1999).

Functional telomere HR closely resembles 'Alternative Lengthening of Telomeres' (ALT)—a dysfunctional form of HR that maintains telomere length in ~10% of cancer types (Henson et al. 2002). Recently a functional form of ALT (ALTn) was described for the first time and found to exist in ~10-15% of normal somatic and germ cells of mice and humans (Neumann et al. 2013; Pickett and Reddel 2012). Normal human fibroblasts *in vitro* can activate ALTn in response to experimentally induced DNA breaks (Berardinelli et al. 2010), and cancer cells that normally utilize telomerase to maintain aberrant cell proliferation, can switch to ALT in the presence of telomerase inhibiting pharmaceuticals (Degryse et al. 2012; Armanios 2012; Agrawal, Dang, and Gabrani 2012; Hu et al. 2012). ALTn occurs in the Iberian shrew *Sorex granarius* (Zhdanova et al. 2014), naked mole rat (E. B. Kim et al. 2011), and marsupial species of the family *Dasyuridae*, including the Tasmanian Devil *(Sarcophilus harrisii)*, which are known for their relatively low metabolic rates compared to eutherian mammals (Bender et al. 2012).

The view of ALT as only dysfunctional, may have hindered its detection in normal cells, however semantics may also have contributed—processes and phenotypic characteristics of ALT appear to have been described in normal cells for more than a

decade, but were given different names; e.g., ‘telomere trimming’ (Pickett et al. 2009), ‘rapid telomere deletion’ (Watson and Shippen 2006)), or “capped” and “uncapped” telomere states (Blackburn 2000; Cesare and Karlseder 2012), ‘recombination telomere elongation’; (Eckert-Boulet and Lisby 2010). I use the term ‘normal ALT’ (ALTn) hereafter for normal HR and to be consistent with recent publications (Neumann et al. 2013; Zhdanova et al. 2014; E. B. Kim et al. 2011).

In summary; the Telomere Hypothesis of Stress and Aging predicts telomere length is dynamic, varies relative to environmental stress exposure, and these length changes serve to facilitate pleiotropic changes in gene expression that function to increase resilience to environmental stress. Evidence indicates a functional form of ALT is likely to facilitate these TL dynamics. Variation in ALT capacity and activity are likely associated with epi-genetic factors or ‘epi-alleles’ which further regulate and modify gene expression patterns. Although ALT is generally thought of as a recombination mechanism, recent evidence indicates ALTn may function in a coordinated manner with telomere transcribing RNA (TERRA).

Telomere Transcription (TERRA) and Normal ALT

The source of telomeric repeats used to lengthen telomeres by ALTn may be derived from transcription by telomeric RNA polymerase II (TERRA) (Episkopou et al. 2014). TERRA occurs in all mammals studied to date (including humans), most yeast (Luke and Lingner 2009), and possibly birds (Solovei, Gaginskaya, and Macgregor

1994). TERRA occurs on nearly all chromosome ends (de Silanes et al. 2014), in somatic and germ cells of mammals (Maicher, Lockhart, and Luke 2014), regulates telomere heterochromatin state, protects telomeres from end to end fusions during telomere truncation events (de Silanes et al. 2014), and facilitates both TL extensions and truncations (Pfeiffer and Lingner 2012; Deng et al. 2009). TERRA also helps coordinate ALTn (Luke and Lingner 2009) and telomerase activity (Cusanelli, Romero, and Chartrand 2013).

The Three 'Telotype' Model of Telomere Function

Telomeres can be classified as one of three 'telotypes'—ALTI, ALTII, or telomerase (TERT). Normal ALT1 cells have short telomeres, ALTII have long telomeres, and TERT has intermediate TL. Cells can progress from the ALT1 to ALTII telotype, but not all of them do (Makovets, Williams, and Blackburn 2008; Lundblad and Blackburn 1993).

Human fibroblasts using ALTn have more relaxed chromatin and increased TERRA expression compared to TERT telotypes (Episkopou et al. 2014). TERRA protects uncapped telomeres, facilitates chromatin remodeling, the formation of telomere loops ('t-loops'), protection of chromosome ends (Okamoto et al. 2013), prevents apoptosis (Maicher, Lockhart, and Luke 2014; Ng et al. 2008; de Silanes et al. 2014), and promotes the formation of extra-chromosomal t-circles (Pfeiffer and Lingner 2012). ALT telotypes possess large numbers of t-circles relative to TERT telotypes, and these extra-

chromosomal DNA circles are likely to facilitate telomere re-lengthening in ALTII telotype cells (mammals; Henson et al. 2009 and *K. lactis*; Groff-Vindman et al. 2005).

The telotype is dependent upon epigenetic factors—in human cells, lower methylation of sub-telomere DNA and histones increases TERRA and vice versa (Arnoult, Van Beneden, and Decottignies 2012). Mouse embryonic stem (ES) cells genetically deficient in DNA methyltransferase (DNMT1 and DNMT3a/b) resemble ALTII telotype cells, having lower sub-telomere methylation, increased ALTn activity, long telomeres, and reduced TERRA activity (Gonzalo et al. 2006). In human cells, ALT telotypes have lower sub-telomere methylation and higher TERRA activity than TERT telotypes (Ng et al. 2008). Increased methylation of human sub-telomeres limits TERRA activity, while sparse or heterogeneous sub-telomere methylation is associated with upregulated TERRA and ALT activity (Ng et al. 2008; Porro et al. 2010).

Telotype-Mediated Stress Resilience

Switching from the TERT telotype to ALTn in response to environmental stressors allows some yeast colonies to survive stress exposure (Lundblad and Blackburn 1993; J. Prescott and Blackburn 1997) and is associated with ~120 changes in gene expression, collectively known as the "telomerase deletion response" or (Nautiyal, DeRisi, and Blackburn 2002). Genes of the TDR form nine functional clusters related to energy production and storage, reproduction, mitochondrial proliferation, cell growth, ribosomal biogenesis, and transcription (Nautiyal, DeRisi, and Blackburn 2002; Conomos, Pickett, and Reddel 2013; reviewed in Ottaviani, Gilson, and Magdinier 2008).

Both ALTI and ALTII telotypes in yeast have enhanced expression of genes promoting meiotic recombination and switching from anaerobic to aerobic respiration (MSC1 and HXK1) (Nautiyal, DeRisi, and Blackburn 2002). In ALTI cells, expression of TDR genes is maximized for six of the nine TDR gene clusters (Nautiyal, DeRisi, and Blackburn 2002). Half of TDR genes contain stress response elements (STRE)—under conditions of caloric restriction, transcription factors MSN2/MSN4 bind STRE and modulate reproductive effort and longevity trade-offs. The ALTI telotypes have enhanced MSN2/MSN4 expression, reduced reproductive effort and increased longevity and vice versa (Fabrizio et al. 2004; Medvedik et al. 2007).

Sirtuins (protein deacetylase enzymes) directly link telotype switching with stress resilience in yeast and possibly mammals (Makovets, Williams, and Blackburn 2008). In yeast, Sir2 maintains telomere heterochromatin, but in response to stress it re-localizes to DNA damage sites, decreasing telomere heterochromatin and increasing gene expression (Medvedik et al. 2007). Sir2 levels are regulated by NAD—Sir2 facilitates relocation of MSN2/MSN4 from cytoplasm to the nucleus where it increases PNC1 activity, cellular respiration, and NAD biosynthesis. In turn, NAD levels regulate Sir2 (Medvedik et al. 2007).

In mouse embryonic stem cells (ES), the Sir2 homologue SIRT1 has similar effects (Oberdoerffer et al. 2008). The sirtuin SIRT6 has similar function to SIRT1, but occurs only in the nucleus, and when SIRT6 levels are upregulated, telomeres are truncated, recombination events increase (Cheng et al. 2012; Tennen et al. 2011), insulin-like growth factor (IGF1) is down-regulated, cellular metabolism is slowed, and

longevity is increased in males by 15% (Kanfi et al. 2012). Resveratrol (a synthetic activator of SIRT1) increased lifespan of obese mice 44% (Minor et al. 2011).

In humans, SIRT1 is positively is associated with reduced acetylation of histone H3-K9 and increased tri-methyl H3-K9 at the TERT gene, which resulting in reduced TERT expression (B. Zhang et al. 2014). Increased oxidative stress recruits DNA methyltransferase (DMNT1) and SIRT1 to sub-telomeres, remodeling chromatin in response to stress (O'Hagan et al. 2011). In human cells *in vitro*, switching between ALT and TERT telotypes is mediated by Werner protein (WRN) (Siddiqa et al. 2012), a RecQ helicase and exonuclease directly involved in telomere length regulation (Multani and Chang 2007). Increased expression of WRN results in increased cellular stress response, phosphorylation of ATF2 (a positive regulator of transcription and positively related to TERRA), and open chromatin structure (Tudor et al. 2009). Both stress-induced senescent and replicative senescent cells in the GI tract of mice resemble ALTI telotypes, possessing reduced heterochromatin, lower methylation of sub-telomere, increased recombination and TERRA (Hewitt et al. 2012). Thus, mechanisms that facilitate resilience to environmental stressors are evolutionarily conserved (e.g., occurring in budding yeast, mice and humans)—exposure to environmental stress triggers the co-location of chromatin remodeling proteins (e.g., surtuins, DNA methyltransferases) at telomeres and sub-telomeres resulting in pleiotropic changes in gene expression conferring stress resilience, including those involved in telomere length maintenance (e.g., TERT).

Heritability of Telomere Length

Telomere length may be largely determined by environmental influence on epigenetic signatures. A recent study of over 19,000 humans from six populations, estimated telomere length heritability to be 70% with TL of spouses having higher correlation coefficients than siblings in some populations, which increased with time spent living together (Broer et al. 2013). Sperm TL increases with age in humans and is inherited by sons (De Meyer et al. 2007; Eisenberg, Hayes, and Kuzawa 2012; Broer et al. 2013). Few common variants of single-nucleotide polymorphisms (SNP), including genome-wide association studies, have been identified (Blackburn 2011), and only those associated with the TERC locus (Soder et al. 1997) have been replicated reliably (Codd et al. 2010; D. Levy et al. 2010; Prescott et al. 2011). Common epigenetic SNP variants, not uniquely associated with telomeres, are correlated with TL (Kim et al. 2012).

Epigenetic signatures affecting telomere length are most readily acquired during key sensitive periods—1) gamete formation (D. T. Eisenberg and Kuzawa 2013, Price et al. 2013); 2) shortly after zygote formation (Daxinger and Whitelaw 2012); and 3) in utero in response to maternal chemical signals (S. Entringer et al. 2011; Sonja Entringer, Buss, and Wadhwa 2012; Sonja Entringer et al. 2013).In chickens, embryonic exposure to glucocorticoids (CORT) caused shortened TL and altered stress responses as juveniles (Mark F. Haussmann et al. 2012). The sperm of mice exposed to either chronic stress in adolescence or adulthood had extensive epigenetic reprogramming, which was inherited by male offspring, resulting in widespread transcriptional changes in stress regulatory pathways (Rodgers et al. 2013). In humans, sub-telomeric methylation patterns associated

with TL are heritable, despite only 5% of genome-wide DNA methylation being heritable (Blasco 2007; S. Kim et al. 2012).

The trans-generational inheritance of epi-alleles increases penetrance with subsequent environmental reinforcement. Epi-alleles associated with TL are heritable for at least two generations in mice (Franklin et al. 2010; Margueron and Reinberg 2010). In the case of the mouse A^{vy} epi-allele, which is associated with long TL and can be acquired by adults exposed to methyl donor supplemented diets, its penetrance increases with each subsequent generation's exposure to methyl donor supplements and reversed in one generation with a control diet (Cropley et al. 2012). Epigenetic signatures can override genetic determinants of TL—TERT haplo-insufficient mice *(Tert+/-)* produce an increasing proportion of offspring with short TL and maintain TL without telomerase (Hathcock et al. 2002), yet restoration to the wild-type genotype *(Tert+/+)* fails to restore TL after more than six generations (Chiang et al. 2010).

Exposure to environmental stress during early life, establishes a strong predisposition for a telomere length set point and epigenetic profile (Margueron and Reinberg 2010). Pre-natal maternal psychosocial stress has been associated with shorter TL in newborns (Sonja Entringer et al. 2013). However, epigenetic changes associated with short TL can be induced throughout life. Global epigenetic signatures of monozygotic twins are similar at birth, but can differ in their DNA methylation profiles of telomeres later in life (Fraga et al. 2005). These differences increase with age, time spent apart, and lifestyle differences (Fraga et al. 2005). Children at risk of early life stress had short TL relative to low risk children, but TL increased with parental care

(Asok et al. 2014). Other examples of environmental stressors that have been negatively associated with TL in humans are childhood maltreatment (Tyrka et al. 2012; Schury and Kolassa 2012), childhood institutional care (Drury et al. 2011), chronic psychosocial stress exposure in adults (Epel et al. 2004; S. Entringer et al. 2011), social resources and physiological resilience in elderly adults (Zalli et al. 2014), and increased risk of post-traumatic stress disorder (PTSD) (Ventura-Junca and Herrera 2012). Experimentally elevated stress hormone levels (CORT) in humans are also negatively associated with TL (Ventura-Junca and Herrera 2012).

The cause of telomere shortening in response to stress is generally believed to be due to accelerated cell replication and equivalent to accelerated aging (e.g., Epel et al. 2004). For example, mothers with chronically ill children and shorter TL were projected to have a 10 year decrease in life expectancy (Epel et al. 2004). However, more recently these authors speculated the effect might be due changes in gene expression and switching to a telomerase independent TL maintenance mechanism;

> *"These compounds (CORT and other biochemical factors) help to mediate an appropriate response to short-term stress. But when overproduced for months or years, they can alter gene expression, probably with deleterious effects. In the laboratory, the same factors (psychosocial stress) can shorten telomeres —in the case of cortisol, by reducing the activity of telomerase. It is likely that the pathways that mediate alterations to gene expression interact with those affecting*

telomere maintenance, although this has yet to be explored" (E. H. Blackburn and Epel 2012).

This latter view is consistent with the THSA, which predicts short telomeres associated with environmental stress are functional, serving to alter gene expression patterns, telomere chromatin structure and the length maintenance mechanism (i.e., ALTn).

Mechanisms of Stress and TL Interaction

Molecular mechanisms directly link environmental stress exposure to telomere shortening. In humans, when GR (CORT receptor) is activated by a ligand (e.g., CORT), apoptosis signaling is interrupted (Sengupta 2001)., Gene expression changes that modulate cellular sensitivity to CORT are activated (e.g., $p16^{INK4a}$ (Roca, Kypta, and Vivanco 2003). If the exposure to stress is short in duration (acute) then $p16^{INK4a}$ stalls the cell cycle in G1 phase, however if the stress is chronic, $p16^{INK4a}$ irreversibly triggers cellular senescence (Roca, Kypta, and Vivanco 2003). This temporary interruption of the cell cycle under conditions of acute environmental stress facilitates recruitment of chromatin remodeling factors to telomeres (e.g., ATP-dependent nucleosome remodeling complexes, HDAC; Serrano, M., Hannon, G. J. & Beach, D; reviewed in, Zhang and Dean 2001; Nguyen and Crowe 1999).. Chromatin remodeling represses transcription of genes needed for cell cycle progression (Zhang and Dean 2001), increases the

transcriptional activity of GR (Singh, Coe, and Hong 1995), and silences transcription of the catalytic subunit of telomerase (hTERT) preventing telomere length extension by telomerase (Bazarov et al. 2010; reviewed in, O'Sullivan and Almouzni 2014). When an acute stressor (<24 hours) is lifted, the cell cycle resumes, however if the stressor is chronic, telomerase activity may be suppressed long after the stress is lifted (29-55 days) (Bazarov et al. 2010). In this case, aa portion of cells will re-lengthen telomeres by an ALTn/TERRA mechanism (García-Cao et al. 2002; Bazarov et al. 2010) . Thus, telomere shortening in response to stress exposure is likely to be related to remodeling of the chromatin architecture to facilitate stress coping genetic pathways, but when the stress is lifted telomeres may be re-lengthened by ALTn mechanisms.

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CHAPTER 3. TELOMERE LOSS AND LIFESPAN RELATIONSHIPS DIFFER BETWEEN BIRDS AND MAMMALS

Abstract

Telomeres are the repetitive sequences of nucleotides terminating eukaryotic chromosomes. Telomeres are hypothesized to shorten with each cell replication and although can be repaired by the enzyme telomerase, this generally occurs only in periods or cells replicating at high rates (i.e., organismal growth, lymphocyte replication). Thus, the rate of cell replication is predicted to result in higher rates of telomeres shortening with age (TROC). Among species of birds and mammals, species with shorter lifespans have higher mass specific metabolic rates. However, birds live several times longer for their specific metabolic rate than mammals. Whether or not TROC differs between birds and mammals is unknown. The goal of this study was to examine TROC among bird species from previously published studies and compare these to mammals. Results indicate that TROC is higher for shorter lived birds and mammals, but for a given lifespan, birds had lower TROC than mammals. The ability of birds to live longer for their specific metabolic rate than mammals may be related to lower TROC. It was recently demonstrated birds replicate telomeres with rolling circle replication—a mechanism that is likely to be rare or infrequent in mammals and may account for their lower TROC.

Introduction

Telomeres are repetitive, non-coding sequences of DNA forming the ends of linear chromosomes (Blackburn 1991). The Telomere Hypothesis of Cellular Aging (THCA) is the predominant mechanistic theory of aging (Harley et al. 1992) and predicts telomeres shorten with each cell division until reaching a 'critical length' or 'Hayflick Limit' (Hayflick 1965), at which time replication ceases, and cells senesce or die (M. Mikhelson and A. Gamaley 2013). Telomere shortening is thought to protect an organism from the proliferation and accumulation of damaged DNA, with the trade-off being that over time, non-replicating cells accumulate as do the phenotypic characteristics of organismal aging. Rates of telomere shortening (TROC) are thus predicted to be positively negatively related to cell replication rates (Allsopp et al. 1995). Cell replication rates are positively related to organismal metabolic rate (Mason and Rathmell 2011; Slavov, Botstein, and Caudy 2014), and is therefore predicted to be negatively related to the rate of telomere shortening.

The rate of living theory (ROLT) describes the negative relationship between species' mass and lifespan—heavier species live longer and have lower specific metabolic rates than lighter species (Rubner 1908; Pearl 1928). Kleiber (1947) formalized theoretical relationships between basal metabolic rate (BMR), mass, and lifespan based on slopes *(B)* of power functions ($Y=AX^B$, i.e., 'Kleiber's Law'). Despite consistent support for these relationships there are some noteworthy discrepancies—the mass-specific metabolic rate of birds (BMR $^{-1}$ unit mass, 'MbBMR') is more than 3 times that of mammals for a given lifespan (Glazier 2008; Munshi-South and Wilkinson 2010; Vleck, Haussmann, and Vleck 2007). How birds achieve their higher specific metabolic

rates without a proportional decrease in lifespan has been an enduring and most intriguing mysteries in animal physiology.

However, recent observations indicate (Ch. 2 & 3) birds possess extra-chromosomal t-circles, or circular forms of telomeric DNA, and these are sites of rolling circle replication of telomeres. This provides a mechanism to rapidly truncate and re-extend terminal telomeres in non-replicating cells. Also, it appears birds have a much greater capacity for this than mammals and that the amount of telomere replication that occurs via rolling circle replication is related to environmental stressors (Ch. 4). This difference between birds and mammals in general, could reduce telomere shortening with age, which could potentially increase their lifespans for their specific metabolic rate relative to mammals.

Maximum lifespan for a species is negatively related to rates of telomere shortening in mammals (M. F. Haussmann et al. 2003), but this relationship is unknown for birds (but see; (Dantzer and Fletcher 2015). Only a few species were available when TROC was initially compared between birds and mammals, and at the time, it appeared they might have similar rates of TROC (M. F. Haussmann et al. 2003). Dantzer and Fletcher (2015) recently addressed this question for a larger sample of bird species and concluded TROC rates were the similar between birds and mammals. However, this latter study did not include juvenile age classes, where TROC is highest. Thus, to achieve better resolution of TROC over the full lifespan of birds and mammals, it is necessary to readdress this question.

The primary goal of this study was to test the prediction that birds have lower rates of telomere loss with age for their lifespan and mass than mammals. Another goal

was to assess the weight of evidence that rates of telomere loss with age reflect metabolic rate. Data were compiled from existing studies of telomere length and age relationships from 16 bird and 8 mammal species. Results indicate birds lose telomeres with age at slower rates than mammals for a given lifespan and mass, which supports the hypothesis that birds possess a mechanism to maintain lower rates of telomere loss than mammals despite their higher metabolic rate. Mass appears to reflect a limit to the total number of telomere sequences potentially lost over a species' lifespan, but birds achieve lifespans comparable to the much heavier mammals by reducing rates of telomere loss for their mass.

Methods

MLSP and mass data

Maximum lifespan (MLSP) and body mass (M) data were gathered from published studies (Appendix I). These data were compared to a those in the Anage database to determine how well they represented birds in general (de Magalhaes and Costa 2009).

Phylogenetics

To assess the potential for phylogeny to confound interpretations of TROC, MLSP, and M relationships, correlations were examined among TROC, MLSP, M, and radiation dates of the avian orders based on molecular clock methods (Pacheco et al. 2011).

Phylogeny was not analyzed statistically for mammals owing to the small sample of available species.

TROC Data

Telomere rate of change estimates were obtained from published studies in one of two ways—directly from text or tables, and/or extracted from figures with image analysis software (Engauge Digitizer v. 5.1). Bird telomere length and age data extracted from figures are illustrated in Fig. 1.

Studies were included if telomere length was quantified with the TRF assay as opposed to QPCR, which does not distinguish between terminal and interstitial telomeres. Many organisms and birds in particular, possess large and highly variable amounts of 'interstitial' telomeres bordered by genomic DNA (Delany, Krupkin, and Miller 2000; Christopher G. Foote, Vleck, and Vleck 2013), which can cause estimates of telomere length to differ significantly between QPCR and the TRF assay (Young et al. 2013).

One study (Pauliny et al. 2006) used the software program TELOMETRIC (Grant et al. 2001), which is known to over-estimate telomere length (Goehring et al. 2014;

Salomons et al. 2009; Mark F. Haussmann and Mauck 2008), but is unlikely to affect estimates of TROC.

Data analysis

Telomere length rate of change (TROC) was quantified as the slope of the least squares regression line from Model I regression analysis of age *(x)* in years, versus telomere length *(y)* (M. F. Haussmann et al. 2003).

Relationships between TROC, MLSP, and M were modeled with power functions following the form: $Y = AX^B$. Models were fit to untransformed data.

All analyses were conducted in (*GraphPad Prism for Windows* (version 6.04) 2014).

Results

Descriptive statistics

Mean MLSP of bird species included in our sample differed from the Anage database (Anage: 17±0.5, t=5.0, df=466, P<0.0001), however the range of ages sampled were similar (this study: 6-60yrs, Anage:3-70yrs). Of the 450 bird species for which MLSP was available in the Anage database, median MLSP was 13 yrs, with 75% of species having MLSP ≤ 23 yrs, compared to a median MLSP of 29 yrs, with 75% of species ≤ 36 years for our sample species.

Mean body mass of bird species included in this study was similar to those in the Anage database (Anage: 948±134 g; t=0.42, df=1100, P=0.672).

Body mass (Mb) differed between classes (bird: 1412±574 g; mammal: 143205±81809; t=2.4, df=26, P=0.028), but mean MLSP was similar (bird: 29±3.1 yrs; mammals 41±11 yrs; t=1.4, df=26, P=0.163).

Effects of phylogeny

Maximum lifespan and M were positively correlated with radiation dates of the avian orders (MLSP: R^2=0.51, P=0.018; Mb: R^2=0.49, P=0.012), but TROC was not (R^2=0.02).

TROC and maximum lifespan relationships

The rate of telomere shortening decreased with increasing MLSP for birds and mammals and differed between classes (Table 1, Fig. 2a).

TROC and mass

The relationship between body mass (M) and TROC was stronger for birds than mammals (Table 1, Fig. 2b). Scaling factors were similar, but *A* differed—birds had lower TROC than mammals for a given M (Table 1).

L-TROC and body mass

Lifetime TROC and M relationships were similar between classes (Table 1, Fig. 2c).

Discussion

Birds lost telomeres with age in the majority of species (Fig. 1, Appendix II), although the average proportion of TROC variability accounted for by age was only about 30%. Birds had lower TROC for given lifespan than mammals. Lifetime TROC for birds and mammals were similar when accounting for differences in mass between classes (Fig 2c). Taken together, these results suggest lower TROC of birds may facilitate longer lifespans of birds for their mass than observed in mammals. Also, results do not support the THCA as birds are expected to have higher TROC than mammals given their higher specific metabolic rate—higher metabolic rates increase oxidative stress and telomere shortening (Caldini et al. 1998; Richter and von Zglinicki 2007).

Recent evidence indicates birds possess a mechanism of replicating telomeres from circular telomeric DNA motifs outside of chromosomes that occurs at much higher levels than in observed in mammals (Ch. 2; Henson et al. 2005, 2009). It is hypothesized this mechanism, resembling a normal form of ALT (reviewed in Ch. 1) facilitates telomere length changes, independent of telomerase, and in non-replicating cells. The function of normal ALT in birds is unclear, but levels were found to increase in response to changes in stress hormone levels in black-legged kittiwakes (Ch. 6), which supports

the hypothesis that normal ALT facilitates changes in gene expression in response to environmental stress, including changes in metabolic pathways and defenses against oxidative damage (reviewed in Ch. 1; Ye et al. 2014). Thus, the presence of normal ALT in birds may mitigate costs of maintaining higher specific metabolic rates than mammals, and ultimately longer lifespans for a given mass.

Phylogeny can impose constraints on phenotypic plasticity and evolvability, but variation among species can also represent a continuum of increasing species radiation over evolutionary time-scales. In the case of birds, lifespan and mass were correlated with phylogeny such that the shortest lived and lightest species were the most recent to diverge. The evolution of smaller mass in birds is believed to have conferred a number of advantages for additional niche exploitation (e.g., increased brain development, migratory behavior, and aerial foraging; reviewed in (Starck 1998)). Functional ALT may facilitate evolution of smaller mass by increasing resilience to environmental stressors.

Table 1. Best-fit values of non-linear relationships between telomere length rate of change per year (TROC), maximum lifespan (MLSP), mass (M); and lifetime telomere loss (L-TROC). P-values are from F-tests comparing the fit of power model parameters ($Y = AX^B$) between taxonomic classes versus a global model and testing the hypothesis that B=0.

			Best Fit			Slope ≠ 0?	Classes Different?
X	Y	Class	R^2	A	B	P-value	P-value
MLSP	TROC	Bird	0.81	3163	-0.93	<0.0001	0.007
		Mammal	0.50	1188	-0.34	0.034	
M	TROC	Bird	0.44	-8324	-1.21	0.023	0.004
		Mammal	0.21	-757	-0.09	ns	
M	L-TROC	Bird	0.52	-1	1.01	0.025	ns
		Mammal	0.35	-1453	0.15	ns	

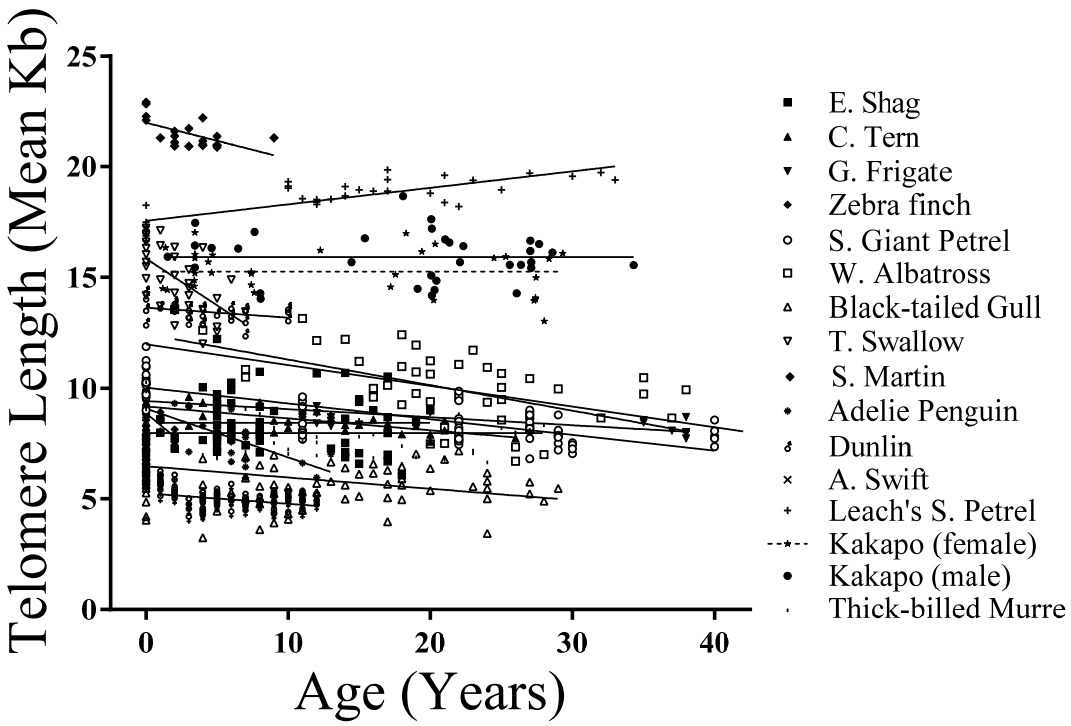

Figure 1. Telomere length change with age (TROC kb yr^{-1}) for fifteen bird species (Model I least squares regression). Data are cross-sectional and extracted from published figures (See Appendix I, for data sources and Appendix II for summary data.

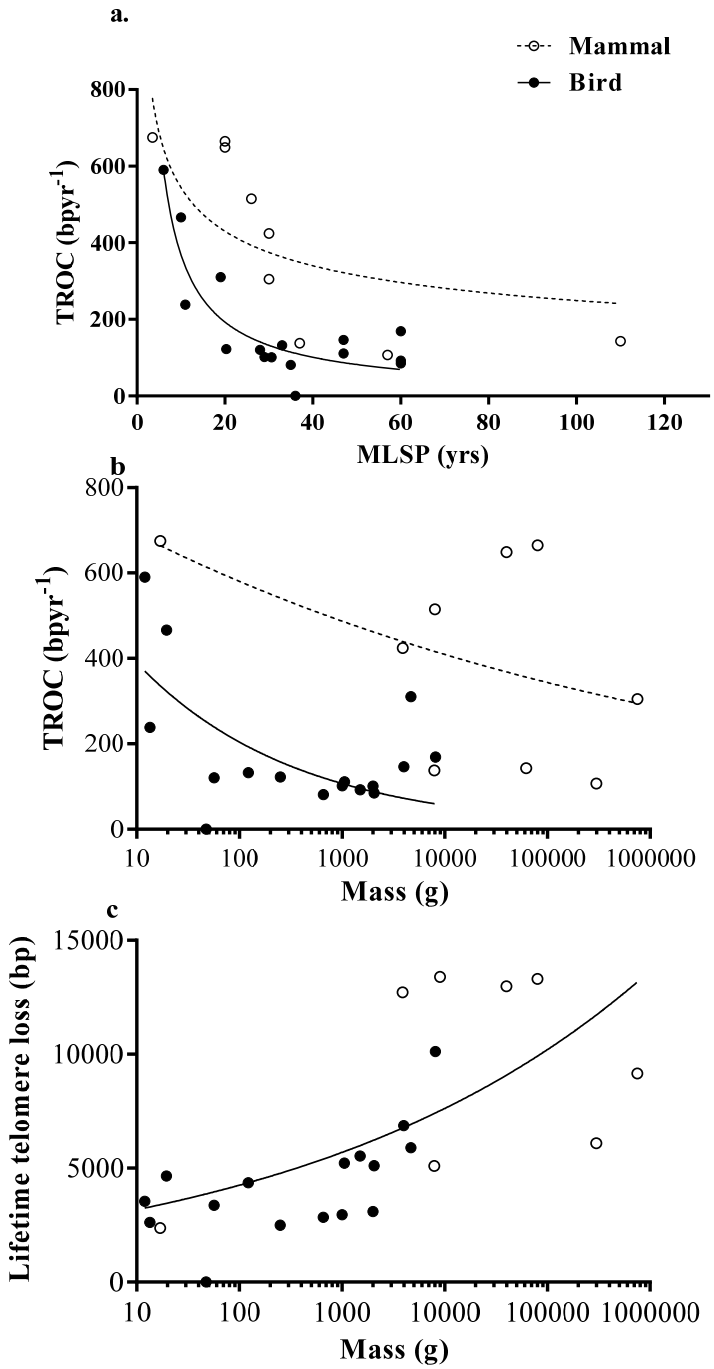

Figure 2. Power relationships ($Y=AX^B$) of; a) telomere rate of change (TROC bp yr^{-1}) relative to maximum lifespan (MLSP), b) mass (M), and c) lifetime telomere loss (L-TROC) relative to mass (M). Bird data are filled circles with solid lines; mammals are open circles with dashed lines. All statistical tests were performed with best fit parameters (see Table 1 statistics.)

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Appendix I. Sources of TROC, maximum age (MLSP), and mass (Mb).

Class	Species	MLSP Source	TROC Source	Mass Source
Aves	E. Jackdaw	(Fransson, T., et al. 2010)	(Salomons et al. 2009)	(de Magalhaes and Costa 2009)
	E. Shag	(Fransson, T., et al. 2010)	(Hall et al. 2004)	(de Magalhaes and Costa 2009)
	C. Tern	(Fransson, T., et al. 2010)	(Bauch, Becker, and Verhulst 2012)	(Nisbet 2002)
	Great Frigatebird	("Longevity Records of North American Birds" n.d.)	(Juola et al. 2006)	(Gauger Metz and Schreiber 2002)
	Zebra Finch	(Holmes and Austad 1995)	(M. F. Haussmann et al. 2003)	(Robertson, Stutchbury, and Cohen 1992)
	Southern Giant Petrel	(C. G. Foote et al. 2010)	(C. G. Foote et al. 2010)	(Obst and Nagy 1992)
	Wandering Albatross	(Weimerskirch et al. 2014)	(Hall et al. 2004)	(Buttemer, Battam, and Hulbert 2008)
	Black-tailed Gull	(Fransson, T., et al. 2010)	(Mizutani 2009)	(John B Dunning 2008)
	Tree Swallow	("Longevity Records of North American Birds" n.d.)	(M. F. Haussmann et al. 2003)	(Robertson, Stutchbury, and Cohen 1992)
	Leach's Storm-petrel	(Carey and Judge 2000)	(M. F. Haussmann et al. 2003)	(Huntington, Butler, and Mauck 1996)
	Adelie Penguin	(David G. Ainley 2002)	(M. F. Haussmann et al. 2003)	(David G. Ainley 2002)
	Sand Martin	("Longevity Records of North American Birds" n.d.)	(Pauliny et al. 2006)	(Garrison 1999)
	Dunlin	("Longevity Records of North American Birds" n.d.)	(Pauliny et al. 2006)	(Warnock and Gill 1996)
	Thick-billed Murre	(Fransson, T., et al. 2010)	(Young et al. 2013)	Kitaysky unpubl. data
Mammalia	*Bos taurus*	(de Magalhaes and Costa 2009)	(Miyashita et al. 2002)	(de Magalhaes and Costa 2009)
	Canis familiaris	""	(Yazawa et al. 2001)	""
	Felis domesticus	""	(McKevitt et al. 2003)	""
	Equus ferus caballus	""	(Katepalli et al. 2008)	""
	Macaca fascicularis	""	(Lee et al. 2002)	""
	Macaca nemestrina	""	(Nasir et al. 2001)	""
	Ovis aries	""	(Shiels et al. 1999)	""
	Mus spretus	""	(Coviello-McLaughlin and Prowse 1997)	""
	Homo sapiens	""	(Broer et al. 2013)	""

Appendix II. TROC, mass, and MLSP values used in analyses. TROC values ("absTROC+min) are reported for convenience of logarithm transformation and are absolute values of TROC plus 75 (the minimum TROC loss, Leach's storm petrel). P values are tests of slope = 0 from linear regression. The "Lit. TROC" values were taken directly from text as reported in literature and where available were used in analyses. When values were not reported in text, TROC values from Figure 1 were used. See Appendix I for list of sources.

Class	Common name	Mass	MLSP	absTROC+min.	Fig. 1 TROC $\overline{x}$ ± sd (n)	P value	Age range	Lit. TROC
Aves	Bank Swallow	13.45	11	238.5	-163.5 ± 232.41 (19)	0.007	1-9	--
	Wandering Albatross	8130	60	168.72	-93.72 ± 144.00 (80)	0.000	2-38	--
	Kakapo (male)	2060	60	85.11	-10.11 ± 122.64 (32)	0.644	1–34	--
	Kakapo (female)	1500	60	92.27	-17.27 ± 93.538 (31)	0.312	1–34	--
	Eurasian Jackdaw	250	20.3	122.61	-47.61 ± 112.83 (111)	0.000	1–14	--
	Southern Giant Petrel	4000	47	146.07	-71.07 ± 57.975 (57)	0.000	1-40	--
	Leach's Storm Petrel	47.4	36	0	74.49 ± 54.554 (32)	0.000	0-33	75
	Great Frigatebird	1054	47	111.1	-36.33 ± 24.816 (32)	0.000	0-40	-36.1
	Dunlin	56.72	28	120.36	-45.36 ± 127.09 (27)	0.075	1-11	--
	European Shag	2000	30.6	101.08	-26.08 ± 237.24 (55)	0.418	1-20	--
	Common Tern	122.33	33	132	-53.78 ± 47.611 (39)	0.000	0-26	-57
	Zebra finch	12	6	590	-529.8 ± 484.58 (13)	0.002	0-3	-515
	Black-tailed Gull	658	35	81.32	-11.08 ± 107.76 (72)	0.386	0-29	-6.32
	Tree Swallow	19.5	10	466	-383 ± 740.08 (21)	0.028	0-7	-391
	Adelie Penguin	4700	19	310	-220.3 ± 239.93 (20)	0.001	1-15	-235
	Thick-billed murres	1000	29	101.9	-26.9 ± 105.23 (59)	0.054	0-28	--
Mammalia	*Bos taurus*	750000	30	305	--	<0.0001	0-18	-230
	Canis familiaris	40000	20	649	--	0.570	1-7	-574
	Equus caballasm	300000	57	106.94	--	--	1-25	-31.94
	Felis catus	3900	30	424	--	--	1-13	-349
	Homo sapiens	62035	110	143.2	--	0.003	2-99	-68.2
	Macaca fascicularis	7913	37	137.7	--	0.010	0-34	-62.7
	Mus spretus	17	3.5	675	--	<0.0001	0-2	-600
	Ovis aries	80000	20	665	--	0.010	1-6	-590
	Macaca nemestrina	8000	26	515	--	--	2-9	-440