TELOMERE CORRELATIONS DURING EARLY LIFE IN A LONG-LIVED SEABIRD

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Jacob Eli Schmidt

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SUPERVISORY COMMITTEE:

Britt Heidinger, PhD
Chair

Wendy Reed, PhD

Kim Vonnahme, PhD

Approved:

3 April 2018
Date

Wendy Reed, PhD
Department Chair

ABSTRACT

Telomere dynamics in blood cells have been linked to aging in a variety of organisms. However, whether blood telomeres correlated with telomeres in other parts of the body is not well known, especially during early life when telomere loss is expected to be most rapid. We investigated this question in embryonic and juvenile Franklin's gulls *(Leucophaeus pipixcan)*. We measured telomere lengths in blood, heart, liver and skeletal muscle tissues at the end of embryonic (n = 31) and post-natal development (n=20). In late-stage embryos, blood telomeres were significantly positively correlated with heart and skeletal muscle, but not liver telomeres. However, at the end of post-natal development, there were no significant correlations among blood telomeres and telomeres in any other tissues. In late-stage embryos, heart telomeres were significantly longer than blood, liver, and skeletal muscle telomeres, but at the end of post-natal development telomere lengths did not significantly differ among tissues.

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1. INTRODUCTION[1]

Understanding the mechanisms affecting longevity, such as telomere dynamics, is of importance to a variety of fields, including evolutionary ecology and biomedicine (Monaghan, 2014). Telomeres are highly conserved, repetitive, non-coding sequences of DNA that together with other proteins form protective caps at the ends of linear chromosomes (Blackburn, 2005). Telomeres enhance genome stability by protecting the coding sequences from loss during normal cell division and by allowing the DNA repair machinery to distinguish double stranded breaks from chromosomes ends (Vleck et al., 2003). Once telomeres become critically short, cells stop dividing and can secrete inflammatory compounds (Blackburn, 2005) and both of these processes are expected to compromise tissue function and contribute to organismal aging (Aubert and Lansdorp, 2008; Carneiro et al., 2016).

In most vertebrates, telomeres shorten with age (Gomes et al., 2010), but loss is expected to be greatest during early life when development is most rapid (Frenck et al., 1998; Heidinger et al., 2012). Early life telomere length has been shown to be positively correlated with lifespan (Heidinger et al., 2012), and telomere dynamics (both length and loss rate) have been found to be better predictors of survival and mortality than chronological age in wild populations (Bize et al.,

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[1] The material in this thesis was co-authored by Jacob Schmidt, Aubrey Sirman, Jeff Kittilson, Wendy Reed, Mark Clark, and Britt Heidinger. Jacob Schmidt had primary responsibility for experimental design, collecting tissue samples, measuring telomeres, conducting statistical analysis and writing and revising this work. Jacob Schmidt and Aubrey Sirman shared animal husbandry duties during this experiment. Jacob Schmidt was the primary developer of the conclusions that are advanced here. Wendy Reed and Mark Clark provided advise on experimental design, expertise working with the study system, and assisted with egg collection. Jeff Kittilson taught Jacob Schmidt how to extract DNA and measure telomeres. Britt Heidinger provided experimental supervision, contributed to revisions and checked the statistics run by Jacob Schmidt.

2009). Thus telomeres are expected to be functionally involved in the aging process and/or serve as biomarkers of an individual's biological age (Bird et al., 2003; Monaghan, 2010; Carneiro et al., 2016).

Telomere length is most commonly measured in blood cells because it is a highly mitotic tissue and it is possible to obtain repeated samples using relatively non-invasive techniques. In mammals, telomeres are typically measured in leukocytes, whereas in birds and reptiles, telomeres tend to be measured in erythrocytes as they are nucleated in these organisms. However, whether blood telomeres are reflective of and correlated with telomeres in other parts of the body is not well understood.

Studies that have addressed this question have typically found positive correlations between blood and telomere lengths in other somatic tissues (Table 1). However, the results are not universal and most of these studies have been conducted on adult humans. Only a single study has addressed this question in a non-mammalian vertebrate and the results were mixed (Reichert et al., 2013). In zebra finches, small songbirds, blood telomere length was positively correlated with spleen and brain, but not with bone marrow, heart, or muscle telomere lengths once a significant outlier was removed (Reichert et al., 2013).

There are reasons to suspect that variation in life-history strategies and developmental stage could impact telomere correlations among tissues. For example, birds tend to grow faster and reach adult size earlier than mammals (Ricklefs, 2010; Swanberg et al., 2010), which could lead to greater variation in proliferation rates and differences in telomere lengths among tissues. However, to date, no studies have investigated whether blood telomeres are correlated with other tissues in developing young in vertebrates other than in mammals. Here we investigated whether blood telomeres were correlated with other somatic tissues including: heart, liver, and skeletal

muscle in embryonic and juvenile Franklin's gulls *(Leucophaeus pipixcan),* a relatively long-lived seabird (Burger and Gochfeld, 2009).

Table 1. Studies examining telomere length correlations between blood and other tissues

Organism	Number	Correlated with blood	Not correlated with blood	Stage	Method	Reference
Humans	N = 87	Fat, muscle, skin	N/A	Adults	TRF	Daniali et al., 2013
Humans	N = 9	Skin, Synovium	N/A	Adults	TRF	Friedrich et al., 2000
Humans	N = 21	Fibroblasts, buccal cells	N/A	Adults	qPCR	Gadalla et al., 2010
Humans	N = 16	Skin, fat	N/A	Adults	TRF	Granick et al., 2011
Humans	N = 29	Cerebellum	N/A	Adults	qPCR	Luken et al., 2009
Humans	N = 12	True vocal folds	False vocal folds, skin, aorta, bone marrow	Adult	qPCR	Thibeault et al., 2006
Humans	N = 110	N/A	Buccal cells	Adult	qPCR	Thomas et al., 2008
Humans	N = 24	CD34+ cells	N/A	Adult	qPCR	Wong et al., 2011
Humans	N = 12	Intercostal muscle	Liver, kidney, heart, brain, triceps, skin, mucosa, fat	Juvenile and adults	qPCR	Dlouha et al., 2014
Humans	N = 22	Skin	N/A	Juvenile	TRF	Okuda et al., 2002
Dogs	N = 83	Fat, muscle	N/A	Adults	TRF	Benetos et al., 2011
Cows	N = 21	Fat, liver, mammary gland	N/A	Adults	qPCR	Laubenthal et al., 2016
Birds	N = 20	Spleen, liver, brain	Muscle, heart, bone marrow	Adult	qPCR	Reichert et al., 2013

2. METHODS

2.1. Study system and sample collection

Research was conducted on a population of Franklin's gulls that breed in north-central North Dakota (Clark and Reed, 2012). Females lay clutches of 1-4 eggs and both males and females incubate the eggs for 23-26 days and feed the chicks for approximately 35 days prior to fledging (Burger, 1974). In May and June of 2014 and 2015, we collected first laid eggs (to control for variation in maternal investment across the clutch) (Engelhardt et al., 2005) and incubated them in the lab at 65% relative humidity. Eggs collected in May were incubated at a 14:10 light to dark photoperiod, and eggs collected in June were incubated at 18:6, to mimic naturally occurring photoperiod cues and to be consistent with previous work conducted in this system (Clark and Reed, 2012).

In 2014, embryos were extracted from eggs near the end of the incubation period (day 18 of incubation) and were promptly weighed and euthanized. In 2015, eggs were allowed to hatch, and the chicks were subsequently reared in captivity in groups of 3 to 4 birds. Chicks were fed an *ad libitum* diet of cat food (Mother and Babycat, Royal Canin USA) from hatching until the end of post-natal development (approximately 40 days post-hatching), at which point they were euthanized. To measure telomere lengths in embryos and chicks, we collected a terminal blood sample and dissected the following tissues: heart, liver and skeletal muscle. Tissue samples were immediately frozen on dry ice. Blood samples were stored on ice for less than 2 hours and centrifuged for 10 minutes at 2000 rpm to separate plasma and red blood cells. All samples were stored at -80^{o}C until being processed for telomere analyses.

2.2. Telomere measurement

DNA was extracted using Macherey Nagel Nucleospin Blood and Nucleospin Tissue kits following the manufacturer's protocol (Genomic DNA from Blood, Genomic DNA from tissues, Macherey Nagel). DNA concentration and purity were assessed with a Nanodrop 8000 (Thermo Scientific) and only samples with 280/260 and 260/230 values above 1.8 were used for telomere measurement.

Relative telomere length was measured using qPCR (quantitative polymerase chain reaction) on an Mx3000P (Stratagene) as described in Cawthon (2002) and modified for use in Franklin's gulls. The relative telomere length (T/S) of the samples was calculated as the ratio of the telomere repeat copy number (T) to that of a single copy control gene (S), relative to the reference sample.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy control gene. We used the following gull specific GAPDH forward and reverse primers (Integrated DNA Technologies): 5'-CGGAGCACCGCTTACAATTT-3' and 5'-GCATCTCCCCACTTGATGTTG-3' respectively. Amplified samples were run on a 3% agarose gel to verify that the amplification was a single product, which yielded a single band at 77 bp as expected. We used the following telomere primers: TEL 1b: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTT-3' and TEL 2b: 5'-GGCTTGCCTTACCCTTACCCTTACCCT-3.

The qPCR reactions for GapDH and telomeres were run on separate plates. In both reactions, the number of PCR cycles (Ct) required for the products to accumulate enough fluorescent signal to cross a threshold was determined. Individuals with short telomeres took longer to cross the threshold (i.e., had higher Ct values) than individuals with long telomeres. All

reactions used 20 ng of DNA in a final volume of 25 µl containing 12.5 µl of SYBER green Master Mix, 0.25 µl forward and reverse primer, 6 µl water, and 6 µl of DNA sample. A negative control of water was run on each plate. All samples were run in triplicate, and average values were used to determine the T/S ratio according to the following formula: $2^{\Delta\Delta Ct}$ formula, where $\Delta\Delta Ct = (C_t^{Telo} - C_t^{GAPDH})$ reference $- (C_t^{Telo} - C_t^{GAPDH})$ (Agilent Technologies, 2012).

In order to assess the efficiencies of each plate, samples were run against a standard curve of 40, 20, 10, 5, and 2.5 ng produced by serially diluting a reference sample. One individual's tissues from 2014 were used as the tissue specific reference samples throughout the entirety of the experiment. Samples were run against tissue specific standard curves. In all cases, plate efficiencies were in the accepted range (i.e. 100+/-15%) and fell within the bounds of the standard curve. Average plate efficiencies and standard errors for GAPDH and telomere plates were 99.02 +/- 3.24, and 90.48 +/- 4.55, respectively. For the 2014 assays, the average intra-plate variation of the Ct values was 0.12% for the GAPDH assays and 1.13% for the telomere assays, and the average inter-plate variation for the ΔCt values was 5.20%. For the 2015 assays, the average intra-plate variation of the Ct values was 0.21% for the GAPDH assays and 1.05% for the telomere assays, and the average inter-plate variation for the ΔCt values was 6.85%.

2.3. Statistical analysis

To determine whether telomere lengths were correlated among tissues within individuals we used Pearson's correlation tests. To examine whether telomere lengths were longer in some tissues than others in embryonic and juvenile gulls, we used a general linear mixed model (lm). We included tissue, sex, season, and season*tissue interaction as fixed effects and individual as a random effect to control for the fact that multiple tissues were collected from the same individual. All terms were retained in the final model to decrease likelihood of type I errors

(Whittingham et. al, 2006). Least squares difference (LSD) tests were used to determine significant differences in telomere lengths among tissues. We also calculated the repeatability of telomere lengths across tissues using the formula $r = s^2_A/(s^2+ s^2_A)$, as described in Lessells and Boag (1987). In order to ensure normality, T/S ratios were natural log transformed for all analyses. All statistical analyses were performed in SPSS (IBM SPSS statistics version 23).

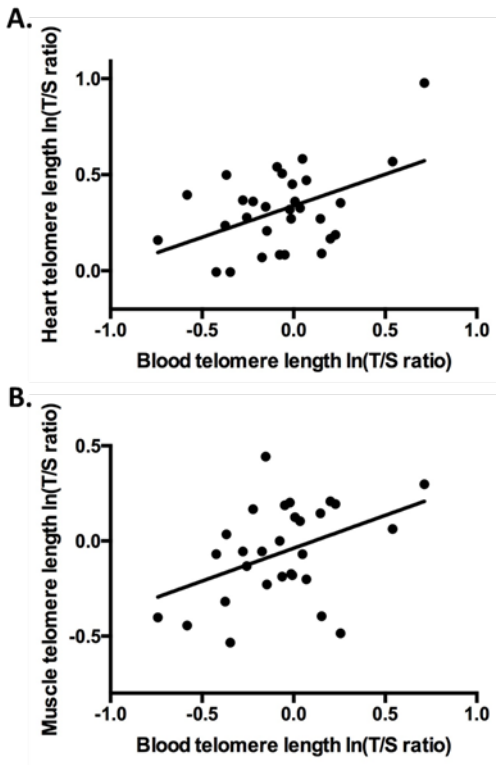
3. RESULTS

2.4. Embryonic tissues

Within individual embryonic gulls, there were significant positive correlations between blood and heart telomeres (r=0.47, p=0.0083, Figure 1A) and blood and muscle telomeres (r=0.42, p=0.0232, Figure 1B), but not among telomere lengths in other tissues (Table 2). Tissue had a significant effect on telomere length ($F_{3,84.661}$= 25.381, $p < 0.001$). Heart telomere length was significantly longer than blood (p<0.001), liver (p<0.001), and muscle telomere length (p<0.001) (Fig. 2A). There were no significant effects of sex ($F_{1,30.406}$=0.321, p=0.728) or season ($F_{1,27.911}$=0.859, p=0.362) on telomere lengths in the different tissues. However, there was a significant interaction between season and tissue; late season embryos had longer liver telomeres than early season embryos ($F_{1,\ 84.661}$ = 6.778, p<0.001). Within individual embryonic gulls, telomere lengths were not repeatable among tissues (r = 0.24918).

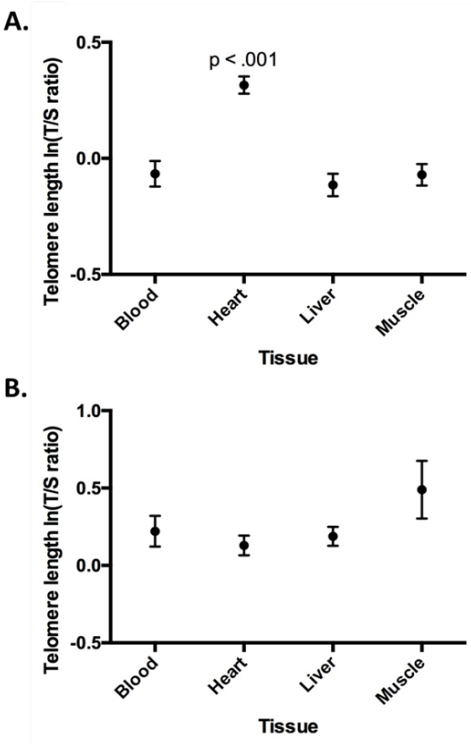
Table 2. Relationships between telomeres in blood, heart, liver and skeletal muscle

Tissue	Stage	Heart	Liver	Muscle
Blood	Embryonic	r=0.470, ***p*=0.008**, n=30	r = -0.25, p =0.189, n=29	r = 0.420, ***p* = 0.023**, n=29
	Juvenile	r=0.220, p=0.359, n=20	r =0.340, p = 0.136, n = 20	r = -0.100, p = 0.669, n =20
Heart	Embryonic		r = 0.040, p =0.830, n=30	r = 0.260, p = 0.501, n=30
	Juvenile		r = 0.190, p = 0.417, n = 20	r = 0.050, p = 0.838, n = 20
Liver	Embryonic			r = 0.190, p = 0.317, n=29
	Juvenile			r=-0.020, p = 0.936, n = 20



Relationship between blood and heart telomere length (r = 0.473, p = 0.008) (A) and blood and skeletal muscle telomere length (p=0.023, r=0.420) (B) in embryonic Franklin's gulls.

Figure 1. Relationship between blood and heart and blood and skeletal muscle telomeres



Telomere length (± SEM, measured by T/S ratio using qPCR) in blood, heart, liver, and muscle of embryonic (A) and juvenile (B) Franklin's gulls. P-values are shown only when $p < 0.05$.

Figure 2. Telomere length in embryonic (A) and juvenile (B) Franklin's gulls

2.5. Post-natal tissues

Within individual gull chicks, there were no significant correlations among telomere lengths in any tissues (Table 2). In addition, there were no significant effects of tissue ($F_{3,54.00}$ = 1.911, p = 0.139, Fig 2B), sex ($F_{1,17}$ = 0.344, p = 0.565), season ($F_{1,17.00}$ = 0.000, p = 0.994), or season * tissue interactions ($F_{54.00}$ = 0.463, p = 0.709) on telomere length. Within individual gull chicks, telomere lengths were not repeatable among tissues (r = 0.0506).

4. DISCUSSION

Blood telomeres are often assumed to be reflective of telomeres in other tissues, but this has seldom been investigated, particularly in young, developing organisms. Here we report in Franklin's gulls, that telomere lengths in blood and other tissues (heart and skeletal muscle, but not liver) were significantly correlated in late term embryos, but not at the end of post-natal growth. Thus in contrast to some prior studies, our results suggest that blood telomeres may not be universally predictive of telomere dynamics in the rest of the body at all stages of development. In addition, we found that heart telomeres were significantly longer than other tissues in embryos, but not in chicks.

Telomeres might be expected to be highly correlated in developing organisms because some evidence suggests that telomerase, an enzyme that adds DNA to the ends of telomeres and recombination mechanisms act to reset telomere length at fertilization (Eisenhauer, 1997; Schaetzlein et al., 2004; Vizlin-hodzic et al., 2009). Once the initial telomere length is set, several factors could contribute to tissue specific differences in telomere shortening rates and affect telomere correlations among tissues. For example, telomerase activity is expected to be down-regulated in most somatic tissues after embryonic development (Blackburn, 2005) and this is known to vary among species (Haussmann et al., 2004). For example, in some long-lived seabirds, such as Leach's storm petrels (*Oceanodroma leucorhoa*) and common terns (*Sterna hirundo*), telomerase activity remains elevated in bone marrow and intestine into adulthood (Haussmann et al., 2007).

Variation in the timing or rate of development could also affect telomere length correlations among tissues. For example, in adult humans, differences in replication rates during development contribute to variation in telomere lengths among tissues (Daniali et al., 2013).

Variation in cell replication rates may weaken correlations between highly mitotic tissues and those that are post-mitotic or experience slower proliferation rates. The lack of correlation we observed in juvenile tissues may be partially due to increasing differences in cell replication rates across tissues. The idea that less replicative tissues retain longer telomere lengths is consistent with our finding that heart telomeres were longer than other tissues in embryonic gulls.

Another factor that could affect telomere length correlations among tissues is exposure to oxidative stress. Oxidative stress, the damage caused to macromolecules by free radicals has been shown to accelerate telomere loss (von Zglinicki, 2002) and these effects can vary among tissues (Sahm and Gümüslü, 2007). If either exposure to or the ability to repair oxidative damage varies among tissues this could affect telomere shortening rates and correlations among tissues. Thus, organisms may incur variable costs of stress exposure among tissues and as a consequence these tissues may age at different rates, which may not always be captured by blood telomeres. For example, Jennings reported that rat pups that were subjected to experimental nutrient deprivation had shorter kidney telomeres, but telomeres of other tissues (brain and liver) were unaffected (Jennings et al., 1999).

Blood telomeres have been shown to be predictive of longevity in humans, other mammals, and in birds (Cawthon, 2003; Fairlie et al., 2015; Bize et al., 2009; Heidinger et al., 2012) and are increasingly used as a marker of biological aging. Our results in Franklin's gulls suggest that telomere length in blood is not always reflective of telomere lengths in other tissues at all stages of development and highlight the need for additional studies examining the causes and functional consequences of these tissues specific differences in telomere length.

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