AGING AND EARLY LIFE STRESS: TELOMERASE DYNAMICS AND THE CONSEQUENCES FOR TELOMERES IN A WILD BIRD

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ABSTRACT

Aging is an underlying risk factor for many major diseases including cancer, cardiovascular disease, and neurodegeneration. Yet we still do not know the full extent of how our bodies age and what determines our lifespan. One mechanism that may play an important role are telomeres, which are protective caps at the end of chromosomes. Telomeres are directly linked to longevity and can be lengthened by the enzyme telomerase. Early life telomere length is critical for lifespan, but we do not know how telomerase performs during this period. Whether variation in telomerase levels can influence telomere length and loss during development with consequences to longevity is still unknown. This thesis focuses on the role of telomerase during post-natal development and its response to stressors and activators with effects on telomeres. Taken together this research enhances our understanding of how telomerase acts and influences telomere during post-natal development.

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CHAPTER 1: INTRODUCTION

Humans have been fascinated for centuries about why organisms age and what is behind the incredible amount of variation in lifespans between individuals. We still do not yet know the full extent of how our bodies age and what determines our lifespan. An underlying risk factor in many major diseases is age, but why aging rates can vary so drastically is still a mystery. Within the last few decades, we have just started to be able to uncover some of the mechanisms that may lead to understanding these questions. One of these mechanisms that may be important are telomeres. They are sequences of non-coding DNA on the ends of eukaryotic chromosomes that enhance genome stability[1]. Telomeres shorten with cell division and stress exposure, and their length is directly linked to cellular aging[1]. When they reach a critically short length, cells become quiescent and can undergo apoptosis[2], and cause reduced longevity and accelerated aging[3,4]. Telomere length in early life is predictive of lifespan[5] and telomeres generally shorten over an organisms lifespan as a result of cellular division and stress[6]. However, the rate of loss is not linear and tends to be greatest during early life[7], possibly due to the high amount of cell replication during post-natal growth.

Interestingly, telomeres can be restored by the enzyme telomerase which adds sequences back onto the chromosomal ends. Because of this, it may be imperative to maintaining homeostatic cellular conditions and genome integrity[8]. It is typically expected to only be active from the blastoderm stage in embryonic development through birth or hatching, and then is downregulated in most somatic tissues as an anti-cancer mechanism. It is also active in stem cells, gametes, and cancer cells, but this varies widely among species. One hypothesis is that the adaption of homeothermy led to the suppression of telomerase and replicative aging in many placental mammals[9]. Mutations in telomerase activity, which is characterized by down-

regulation of telomerase and greater telomere shortening, have been linked to several diseases[10]. However, much of our understanding about the influence of telomerase on telomeres comes from adult model organisms.

In order to fully understand the relationship between telomere length and organismal aging, manipulation of telomeres during early life is imperative. By directly manipulating telomere length we could better understand whether telomeres are causally related to longevity, a biomarker of aging, or both. One way to approach this question is by experimentally activating telomerase, through the use of TA-65. It is an extract of the Chinese root *Astragalus membranaceus* that activates telomerase and has been shown to lengthen telomeres in adult birds and mice[11,12]. Exposure to TA-65 decreases the percentage of critically short telomeres[12,13], and improves some physiological functions such as immune, cardiovascular, metabolic, bone, and inflammatory traits[12–16]. Interestingly, TA-65 treatment has not had any negative side effects or increased rate of cancer incidence[12]. Since telomere loss is expected to be greatest during early life, it is critical to examine the effects of TA-65 on telomeres during post-natal development.

The process of cell division only accounts for a portion of telomere loss and increasing evidence suggests that exposure to stressors also shorten telomeres[4,17–20]. For example, early childhood adversity was associated with shorter telomere lengths in adulthood[19]. Yet why some individuals' telomeres are more resilient to life stress is still unknown. Telomerase may be one of the mechanisms that have evolved to protect against the effects of stress and contribute some of the variation in telomere loss. Interestingly, studies have shown telomerase upregulation in white blood cells in response to environmentally induced stress[21,22], which may mitigate the effect on telomere loss. In vertebrates, glucocorticoid stress hormones are typically elevated during times of stress, and glucocorticoids can have detrimental impacts on telomeres, either directly or by

increasing oxidative stress[20]. In humans, individuals with higher glucocorticoid hormones also have higher telomerase activity, possibly to protect against the negative effects of stress on telomeres[22]. However, to what extent telomerase can buffer telomere loss and contribute to variation in the effects of stress on telomeres is currently unknown.

In order to investigate how telomerase and telomeres vary during development and in response to activators and stress, the following questions were tested. The first question, discussed in chapter 2, examines how experimental activation of telomerase during post-natal development impacts telomeres. The second question, discussed in chapter 3, looks at to what degree telomerase varies in response to stress and impacts telomeres during development. Both of these experiments were conducted on developing house sparrows (*Passer domesticus*). Research was conducted on a free-living population of house sparrows at agricultural research sites at North Dakota State University in Fargo, North Dakota. House sparrows are ideal for answering the study questions because they readily breed in nest boxes, offspring can be easily monitored and sampled throughout development, and chicks grow to adult size within approximately 12 days of hatching. These experimental studies will enhance our understanding of how telomerase activity responds during early life to environmental stress and activation, and its effects on telomere dynamics.

CHAPTER 2: TA-65 DOES NOT INCREASE TELOMERE LENGTH DURING POST-NATAL DEVELOPMENT IN HOUSE SPARROW CHICKS (*PASSER DOMESTICUS*)

Introduction

Aging is an underlying risk factor for many diseases including cancer, cardiovascular disease, and neurodegeneration [23]. However, the pace of aging varies dramatically among individuals and the mechanisms that underlie this variation are poorly understood. One mechanism that may be important in this context is telomeres [1,8]. Telomeres are highly conserved, non-coding DNA sequences that form protective caps at the ends of eukaryotic chromosomes and thereby enhance genome stability. Telomeres shorten during normal cell division and in response to stress [2]. Once they reach a critically short length, cells become quiescent and may undergo apoptosis [2], which is expected to influence organismal aging [3,4]. In support of this, telomeres shorten with age in diverse tissues and individuals with longer telomeres in early life often live longer [5,6].

Although telomeres are often positively associated with lifespan, the degree to which telomeres are causally related to longevity, a biomarker of aging, or both is not well known [24]. To better understand the relationship between telomeres and organismal aging as well as potential trade-offs with other life-history traits, it will be essential to be able to manipulate telomeres, particularly during early life. Telomeres can be restored by the enzyme telomerase, a reverse transcriptase coupled with a RNA template that replaces telomere sequences [1]. Telomerase is active in cells during embryogenesis, however, it is expected to be downregulated in most somatic tissues soon after birth or hatching as an anti-cancer protection mechanism [10]. Interestingly, in longitudinal studies, the rate of telomere loss is not linear and tends to be greatest during early life [7], perhaps because of the high level of cellular replication that occurs

during this stage. Thus, changes in telomerase levels during post-natal development may have particularly critical and persistent effects on telomeres.

Previous research in adult mice, birds, and humans have demonstrated that experimental exposure to TA-65, an extract from the Chinese root *Astragalus membranaceus,* lengthens telomeres [11,12]. In human cells and mice, these positive effects of TA-65 on telomeres appear to be mediated by an increase in telomerase levels [12,25]. In addition, TA-65 exposure also decreases the amount of time required to regenerate keratin-based structures including hair and feathers [11,12]. Importantly, treatment with TA-65 does not appear to be associated with any negative side effects, such as an increased incidence of cancer [12].

While most somatic tissue has very little telomerase activity after embryogenesis, circulating T cells are able to upregulate telomerase activity [26]. Studies have shown that TA-65 increases the proliferative activity in T cells, most likely through the MAPK pathway [25]. Supplementation of TA-65 during adulthood also improved several common biomarkers of aging including immune, cardiovascular, metabolic function, bone density, and reduced inflammation [12,13,25]. However, the mechanism by which TA-65 improves immune function during post-natal development is not well understood. Maintaining immune function through the proliferation of T cells, as well as other biomarkers of aging, is likely to have important fitness consequences.

All prior studies on the effects of TA-65 on telomeres and biomarkers of aging were conducted on adult organisms, yet how TA-65 influences telomeres during early life, at a time when telomere loss is expected to be greatest, is currently unknown. Here we examined how experimental exposure to TA-65 influenced telomeres, the growth of keratin-based structures, and one aspect of cellular immunity, during post-natal development in house sparrow (*Passer domesticus*) chicks. We predicted that chicks exposed to TA-65 during post-natal development

would experience less telomere loss and have longer telomeres at the end of the growth period, as well as faster growth of keratin-based structures and increased immune function relative to controls.

Materials and Methods

Study Organism and Field Site

This experiment was conducted between April and August of 2016 and 2017 on a free-living population of house sparrows (*Passer domesticus*) that breed in nest boxes on agricultural buildings on the North Dakota State University campus. Nest boxes are checked regularly to determine precise egg lay and hatch dates, and chicks are monitored throughout development. At hatching, all chicks were marked with unique colors using Sharpie© markers to identify individuals within the nests.

Experimental Treatment

Two days after hatching, nests were randomly assigned to one of two treatment groups, experimental TA-65 or water control, and all chicks within the same nest received the same treatment. Between days 2 and 10 post-hatching, chicks in the TA-65 group were given daily oral doses of 0.5 mg of TA-65 dissolved in 100 μL of sterile water (n = 22 in 2016 and n = 42 in 2017; for a total of n = 64) following an established protocol [11]. Control chicks received daily oral doses of 100 μL of sterile water (n = 14 in 2016 and n = 43 in 2017; for a total of n = 57).

Growth Measurements and Blood Sample Collection

Growth measurements were recorded on 2, 6, 8, and 10 (± 1) days post-hatching and included body mass, and the following keratin-based structures: culmen length, wing chord, rectrices, and pin feathers. Body mass was measured to the nearest 0.5 g using a pesola. Wing chord, rectrices, and pin feathers were measured to the nearest 0.5 mm, and culmen length was

measured to the nearest 0.1 mm. On days 2 and 10 post-hatching, blood samples were collected from the brachial vein using venipuncture into heparinized capillary tubes. Blood samples were stored on ice for less than 6 hours before they were centrifuged and separated. Red blood cells were then stored at -80°C until further analysis.

Immune Function

During 2017 only, in both experimental and control groups, we measured an aspect of the cellular immune response by testing T lymphocyte proliferation in response to a novel mitogen, phytohemagglutinin (PHA). The initial thickness of the left patagium (wing web) was measured with a micrometer (accurate to 0.01 mm) at day 10 post-hatching by taking three measurements and determining the mean [27]. Following the protocol from [28], 40 µL of the PHA solution was injected intradermally in the center of the left patagium. Approximately 24 hours later, the left patagium was re-measured as described above [28] to assess the effect of the injection on the thickness of the wing web. The difference in thickness (swelling) of the wing web pre-injection relative to 24 hours post-injection is an indicator of immune responsiveness [27], where thicker swelling indicates a more robust cellular immune response.

Telomere Analysis

DNA from nucleated red blood cells was extracted using the Macherey-Nagel Nucleospin® Blood kits following the manufacturer's protocol. DNA concentration was determined using a NanoDrop 8000 (Thermo Scientific ®). Relative telomere length was measured using quantitative PCR (Stratagene Mx3000P), following methods validated for house sparrows and described in previous studies [5,29]. Each reaction mix consisted of 12.5 µL of SYBR green Master Mix, 0.25 µL of forward and reverse primers, 6 µL of water, and 24 ng of DNA diluted in 6 µL of water, for a total of 25 µL in each well. Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was used as a single copy control gene (Integrated DNA Technologies) and qPCR reactions for GAPDH and telomeres were run on separate plates. All samples were run in duplicate. The qPCR thermal profile for GAPDH was 10 minutes at 95˚C, then 40 cycles of 30 seconds at 95˚C and 30 seconds at 60˚C. The qPCR thermal profile for telomeres was 10 minutes at 95˚C, then 27 cycles of 15 seconds at 95˚C, 30 seconds at 58˚C, and 30 seconds at 72˚C.

Ct values were calculated for each sample as the number of PCR cycles (Ct) needed to accumulate enough fluorescent signal to cross a specified threshold (i.e., samples with shorter telomeres take longer to accumulate sufficient product to cross the threshold and have higher Ct values). The average Ct values were used to determine the T/S ratio according to the $2^{\Delta\Delta Ct}$ formula where $\Delta\Delta Ct = (C_t^{Telo} - C_t^{GAPDH})sample - (C_t^{Telo} - C_t^{GAPDH})reference$ [30].

Each plate included a standard curve of 40, 20, 10, 5, and 2.5 ng produced by serially diluting a reference sample in order to assess the efficiencies for each plate and to ensure that all of the samples fell within the bounds of the standard curve. A water sample was also included on each plate as a negative control. One individuals' blood sample was used as a tissue specific reference sample on all plates. The repeatability of T/S ratios was 86% (ICC using a two-way random model with a 95% confidence interval [31]).

Determining Genetic Sex

To determine molecular sex, DNA extracted from red blood cells was diluted using extra pure water to 10 ng/µL. PCR (polymerase chain reaction) was run to amplify the sex chromosomes within the sample. The sex chromosome primers used were as follows: P2: 5'-TCTGCATCGCTAAATCCTTT-3' and P8: 5'-CTCCCAAGGATGAGRAAYTG-3' [32]. The

PCR product was then analyzed by gel electrophoresis and FluorChem FC2 imaging system (Alpha Innotech). Two bands on the gel indicated a female and one band indicated a male.

Statistical Analysis

To determine whether the treatment had an effect on telomere length and the other dependent variables (i.e., growth measurements of mass and keratin-based structures; and wing web swelling), general linear mixed effect models were used. Relative telomere length at 10 days post-hatching is reported as the T/S ratio as described above. The change in telomere length was calculated as the final length minus the initial length adjusted for the regression to the mean (RTM). The RTM equation is

$$RTM = -1\big(p(X_1 - \bar{X}_1) - (X_2 - \bar{X}_2)\big) \ \ where \ \ \rho = \frac{2rs_1s_2}{s_1^2 + s_2^2}$$

where X_1 is T/S ratio at 2 days old, $\bar{X}_1$ is the mean of 2 day old T/S ratios, X_2 is T/S ratio at 10 days old, $\bar{X}_2$ is the mean of 10 day old T/S ratios, r is the correlation coefficient between 10 day old T/S ratios and 2 day old T/S ratios, s is standard deviation, and s^2 is variance.

Three significant outliers were removed from the dataset because the telomere values were greater than two standard deviations away from the mean. Statistical models included treatment, age of the chick at the time of measurement, sex, clutch size, and ordinal date as fixed effects because these variables have been shown to influence telomeres in other studies [5,33,34]. Nest was also included as a random effect to control for the fact that the chicks within a nest were not independent of one another. In models where telomeres (i.e., telomere length at 10 days old and change in telomere length) were the dependent variables, plate number from the qPCR assay was included as a random effect to control for variation among plates. As the effect of the treatment may depend on environmental conditions that could vary by year, initial models also included year and a treatment x year interaction as fixed effects. In models examining the

potential influence of the treatment on growth, a treatment x age interaction and mass were included as fixed effects in order to examine growth trajectories across the study period. Models examining the potential influence of treatment on wing web swelling also included the change in wing web swelling as the dependent variable, and the time between wing web measurements as a random effect to account for any small variation in sampling time. All statistical analyses were performed in RStudio (R Version 3.4.3 with packages lme4 [35], lmerTest [36], ggpubr, ggplot2, nortest). Models, including dependent variable and fixed effects, are presented with the results in Appendix A.

Results

At the beginning of the experiment, neither body size nor telomere length significantly differed between treatment groups (Appendix A). There were no significant main effects of the treatment or year on telomere length at day 10 (Appendix A). There was also no significant interaction between treatment and year on telomere length at day 10 post-hatching ($F_{1,35.9} = 2.76$; $p = 0.105$) (Fig. 1B), however, there was a significant treatment x year interaction effect on the change in telomere length ($F_{1,26.9} = 4.76$; $p = 0.038$), where telomeres shortened more over time in 2017 TA-65 chicks, than 2017 controls or 2016 experimental or control chicks (Fig. 1A). There were no significant main effects of treatment or year on the change in telomere length (Appendix A). Thus, to better understand the interaction between treatment and year on telomeres, data were split and analyzed separately with respect to year (see analyses below).

In both years, the growth trajectories (i.e. slope) of the keratin based structures including rectrices (Fig. 2) and culmens (Fig. 3) were significantly reduced in TA-65 chicks compared to controls (Table A1). In addition, wing chord ($F_{1,460.7} = 3.47$; $p = 0.063$) and pin feathers ($F_{1,162.1} = 3.58$; $p = 0.060$) in TA-65 chicks tended to grow more slowly than controls. There was no

significant effect of treatment on final mass ($F_{1, 21.1} = 0.59$; $p = 0.449$). However, chicks were significantly lighter at the end of the growth period in 2016 than in 2017 ($F_{1,57.6} = 9.10$; $p = 0.004$).

2016 Results

In 2016, there was no significant effect of treatment on telomere length at 10 days old ($F_{1,23.3} = 0.01$; $p = 0.940$). There was also no significant effect of the treatment on the change in telomere length (RTM) ($F_{1,4.55} = 0.04$; $p = 0.851$). TA-65 chicks rectrices had significantly slower growth trajectories (i.e. slope) than controls, but none of the other growth measures were significantly different between treatments (Appendix A).

2017 Results

In 2017, TA-65 chicks experienced significantly more telomere loss than controls ($F_{1,17.0} = 7.04$; $p = 0.017$) and had significantly shorter telomeres at the end of the experimental treatment ($F_{1,53.8} = 8.53$; $p = 0.005$). In addition, TA-65 chicks also had significantly reduced culmen growth trajectories than controls ($F_{1, 286.2} = 9.84$; $p = 0.002$). However, there were no significant effects of the treatment on any of the other growth measurements (Appendix A). There was also no significant effect of the treatment on the measure of cellular immune responsiveness (wing web swelling following a PHA injection) ($F_{1,15} = 0.94$; $p = 0.346$) (Fig. 4).

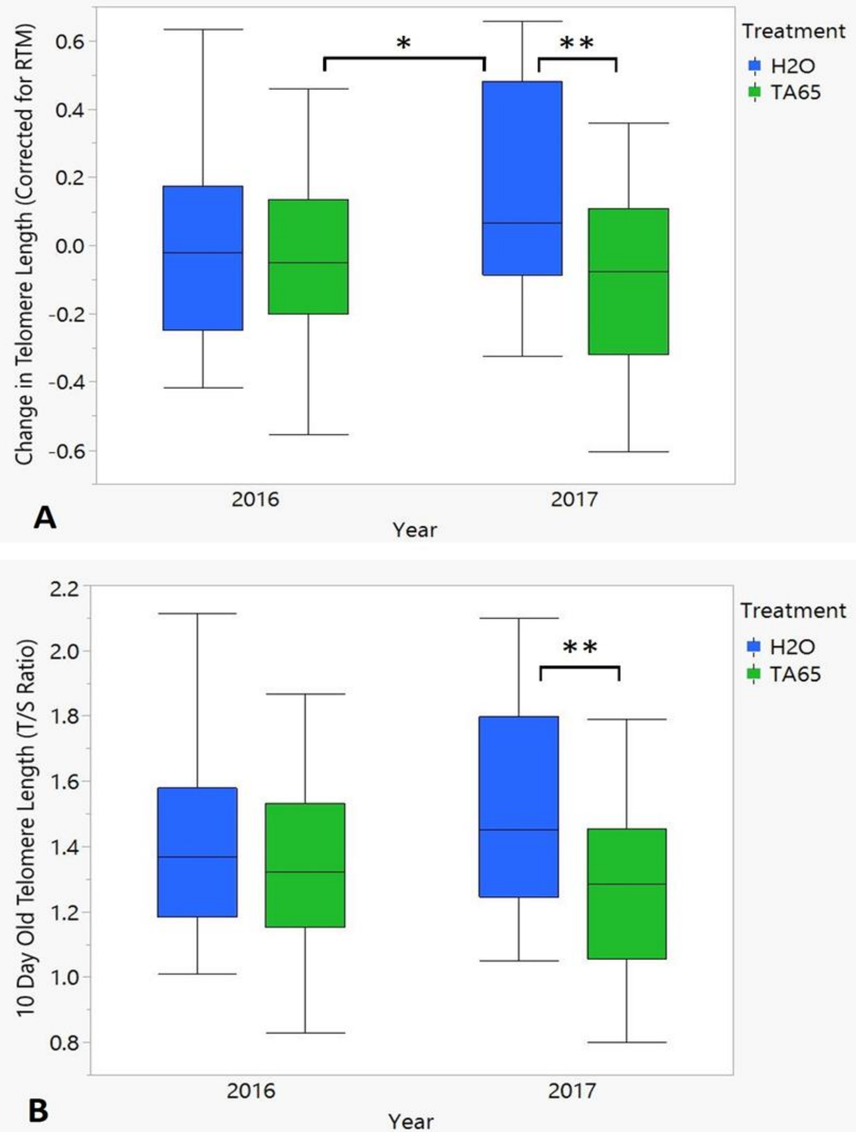


Figure 1: (A) The relationship between treatment and the change in telomere length in house sparrow chicks during post-natal development. More negative numbers denote greater telomere loss. There was a significant treatment x year interaction effect on the change in telomere length ($F_{1,26.9} = 4.76$; $p = 0.038$). In 2017, TA-65 chicks experienced more telomere loss than the controls. Change in telomere length is denoted by 10 days post-hatch telomere length minus 2 days post-hatch telomere length, corrected to the regression to the mean (RTM). (B) The relationship between treatment and 10 day old telomere length at the end of the study period in house sparrows. There was not a significant treatment by year interaction effect on 10 day old telomere length ($F_{1,35.9} = 2.76$; $p = 0.105$). In 2017, the water control and TA-65 treatment were significantly different with TA-65 chicks having shorter telomeres ($F_{1,53.8} = 8.53$; $p = 0.005$). Telomere length is reported by T/S ratio which is calculated using $2^{\Delta\Delta Ct}$ formula (see methods section). Sample size for TA-65 treatment group is *n* = 22 in 2016, and *n* = 42 in 2017. Sample size for H_2O control group is *n* = 14 in 2016, and *n* = 43 in 2017.

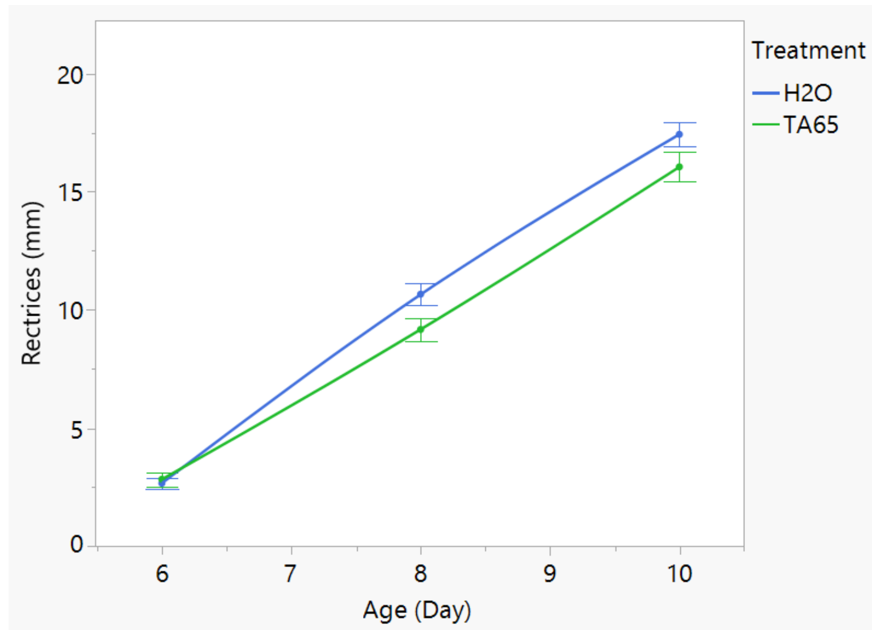


Figure 2: The relationship between treatment and rectrices in house sparrow chicks during post-natal development. Rectrices of TA-65 chicks had significantly less growth than control chicks ($F_{1,203.2} = 6.36$; $p = 0.012$). Error bars were constructed using ± one standard error from the mean. Actual age of chicks varied ± 1 day. Sample size for TA-65 treatment group is $n = 64$, and H_2O control group is $n = 57$.

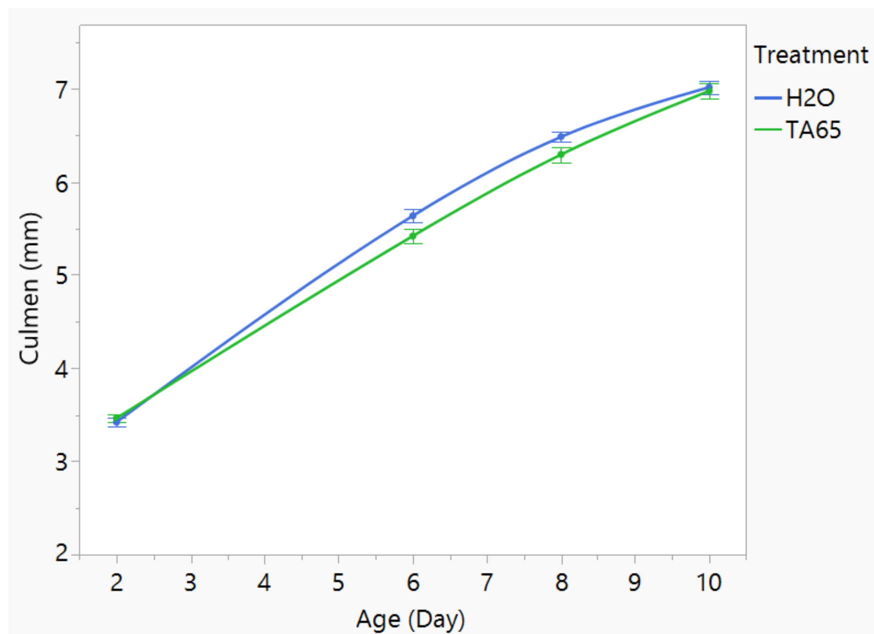


Figure 3: The relationship between treatment and culmens in house sparrow chicks during post-natal development. TA-65 chicks grew significantly smaller culmens than control chicks ($F_{1, 383.8} = 4.8$; $p = 0.029$). Error bars were constructed using ± one standard error from the mean. Actual age of the chicks varied ± 1 day. Sample size for TA-65 treatment group is $n = 64$, and H_2O control group is $n = 57$.

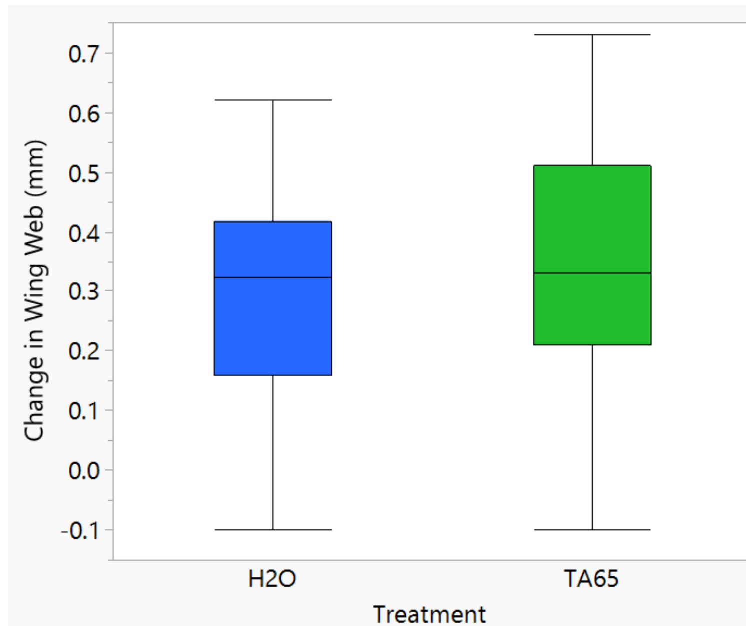


Figure 4: The relationship between treatment and the change in wing web swelling after an injection of PHA, which is a novel mitogen and causes a T lymphocyte proliferation response. There was no significant effect of treatment on wing web swelling 24 hours after a PHA injection ($F_{1,15} = 0.94$; $p = 0.346$) in 11 day old house sparrow chicks. Positive numbers indicate an increase in swelling 24 hours after the injection. This immune response test was only performed in the second year of the study (2017). Sample size for TA-65 treatment group is $n = 37$, and H2O control group is $n = 42$.

Discussion

Previous studies on the effects of TA-65 on telomeres and the growth of keratin-based structures have focused on adult organisms. Importantly, telomere loss is often greatest during development and early life telomere length is predictive of lifespan [5]. Therefore, the effects of TA-65 on telomeres during this life-stage may be particularly important. Contrary to our expectations, chicks exposed to TA-65 did not experience reduced telomere loss during post-natal growth as compared to controls. Instead, in the second year of the study, chicks exposed to TA-65 experienced more telomere loss during development than controls. In addition, in both years, the growth of several keratin-based structures including culmen and rectrices were reduced in TA-65 chicks relative to controls. However, there was no significant effect of TA-65 treatment on immune responsiveness. These results are in contrast to previous studies in adults,

as adult mice, zebra finches, and humans exposed to TA-65 had longer telomeres, greater growth of keratin-based structures, and immune measures relative to controls [11–13].

There are several reasons why the effects of TA-65 exposure on telomeres and the growth of keratin-based structures may differ across life-stages. One possibility is that exposure to TA-65 suppresses endogenous telomerase activity during post-natal development. Although telomerase is expected to be downregulated after birth or hatching, there is evidence that telomerase continues to be expressed even into adulthood in some species [37–39]. Currently it is unknown whether house sparrow chicks continue to express telomerase in somatic tissues during post-natal development. If so, TA-65 may induce the down-regulation of endogenous telomerase activity through a negative feedback mechanism during this time. Alternatively, there may be dose dependent effects of TA-65 on telomeres that differ with respect to life-stage. The TA-65 dose used in this study was modeled after Reichert et al. (2014)[11], which was conducted on adult zebra finches. Another important difference between our study and previous studies on adult organisms is the duration of exposure to TA-65. In the adult studies, individuals were exposed to TA-65 for 2-12 months [11–13], whereas in our study chicks were only exposed to TA-65 for a more limited time period (~8 days) and our results may have differed if the exposure period had been longer. This would not be possible in free-living house sparrow chicks as handling after day 10 can induce chicks to leave the nest prematurely but could be done in a captive study.

The effect of TA-65 on telomere length also differed between years. Importantly, sample sizes differed between years and our sample size was much higher in 2017 than it was in 2016 (n = 36 in 2016 vs n = 85 in 2017). Annual differences in the effects of TA-65 on telomeres could also be due to variation in aspects of environmental quality including food availability and/or weather patterns that affected the development of the chicks. For example, chicks were lighter at

the end of growth in 2016 than in 2017 and it may be that the effects of TA-65 on telomeres and growth are context dependent.

Differences in the effects of TA-65 on the growth of keratin-based structures across life-stages could be due to variation in cell proliferation. Studies that found an increase in the growth of keratin-based structures in adult organisms hypothesized that the increase in telomerase activity may positively affect cell renewal and proliferation capacity [11,12]. Whereas, during early life, the cellular capacity for division may be negatively affected by TA-65 exposure.

Taken together, our results suggest that TA-65 exposure does not increase telomere length or the growth of keratin-based structures during early life as it does in adults. Future research should examine the factors that contribute to the variation in the effects of TA-65 across life-stages including endogenous telomerase expression, dose-dependent effects, and the duration of exposure. Our results provide novel insight into our ability to use TA-65 to manipulate telomeres during post-natal growth. Because telomeres are expected to change most rapidly during early life, and their length at this stage is positively related to longevity, being able to manipulate telomeres during this time period will be essential for examining casual relationships between telomeres and aging.

CHAPTER 3: TELOMERASE ACTIVITY IN RESPONSE TO STRESS AND THE CONSEQUENCES TO TELOMERES IN A WILD BIRD

Introduction

Exposure to chronic stress during development often has long-term consequences for health and longevity [4,17,40,41]. Organisms have evolved protective mechanisms, such as antioxidants, to buffer against the effects of stress. However, these effects can vary dramatically among individuals and the mechanisms underlying this variation are not well understood. One mechanism that may be important in this regard is telomerase, an enzyme that can extend telomeres. Telomeres are non-coding sequences of DNA that protect chromosome ends and are positively related to longevity [6]. Telomeres shorten during cell division and once they reach a critically short length, cells become quiescent and can undergo apoptosis [2]. In zebra finches, individuals with longer telomeres during early life live longer [5]. Besides cellular division, telomeres also shorten in response to stress [18,19], but the rate of stress-induced loss can vary substantially among individuals. Environmental stress has been shown to shorten telomeres and cause greater attrition rates in young and adult organisms [18–20,42]. Although telomeres shorten in response to stress throughout life, the rate of telomere shortening is greater during early life [5,7] and stress experienced at this time may have a particularly large impact on telomeres. There are many routes through which developmental stress exposure could impact telomeres including increases in glucocorticoid stress hormones [20] and oxidative stress levels [43]. But why some individuals' telomeres are more resilient to life stress is still poorly understood.

Although cellular division and stress exposure shorten telomeres, telomere length can be restored by telomerase and other telomere restoration mechanisms. Typically, telomerase is expected to be active from the blastoderm stage through embryonic development and is down-

regulated after hatching or birth in most somatic tissues as an anti-cancer mechanism. It remains active in stem cells, cancer cells, and gametes, however this varies among species. Telomerase has two main subunits: a reverse transcriptase protein (TERT) and the RNA template (TERC). Previous studies have used TERT gene expression as a proxy for measuring telomerase activity.

Interestingly, both correlative and experimental studies have shown that there is some upregulation of telomerase in white blood cells in response to environmentally induced stress, which may buffer the effect of stress on telomere loss [21,22]. Epel et al. (2009) found that glucocorticoid stress hormone reactivity to an acute stress was positively related to higher telomerase activity in humans. However, to what extent variation in telomerase activity affects the impacts of stress exposure on telomeres during this critical time period is currently unknown. Further, the effects of stress-induced changes in telomerase activity on telomeres may vary among tissues, but this is yet to be investigated.

Here, we experimentally manipulated stress exposure in house sparrow chicks (*Passer domesticus*) during post-natal development and examined the effects on telomerase (activity and gene expression) and telomere length in several tissues that vary in proliferation rates including: blood, bone marrow, liver, and pectoralis muscle. We predicted that chicks exposed to experimentally elevated stress that also elevated telomerase would have reduced telomere shortening. We predicted that these effects might vary among tissues, but that telomerase activity and telomere length would be positively related within tissues. This study provides novel insight into the effects of stress exposure on telomerase and telomeres across multiple tissues during post-natal development and will enhance our understanding of the factors that contribute to variation in resilience to developmental stress.

Materials and Methods

Study Organism and Experimental Design

Research was conducted between May and August 2018 on free-living house sparrows (*Passer domesticus*) that breed in nest boxes on agricultural buildings on the North Dakota State University campus. Nest boxes were monitored daily to get precise laying and hatching date of the chicks. Individuals within a nest were marked with Sharpie® markers to distinguish specific individuals throughout post-natal growth. A randomized block design of ten nests were used for this experiment, with two nestlings within each nest randomly assigned to either experimental stress or control treatment groups. Between days 2-10 post-hatching, chicks in the experimental stress treatment were exposed to the daily standardized restraint stress of being placed in a cloth bag for 30 minutes (n = 10). Whereas chicks in the control treatment remained unhandled in the nest during this time (n = 8), and two control nestlings did not survive to collection at 10 days old.

Sample Collection

Growth measurements (mass, culmen, wing chord, tarsus, rectrices, and pin feathers) were collected from all chicks on days 2, 6, 8, and 10 post-hatching. On days 2 and 10 post-hatch, a small blood sample was collected to measure telomeres. To verify that the stress treatment was perceived as stressful by the chicks, on day 10 post-hatching, the chicks were exposed to a standardized restraint stress protocol to measure baseline and stress-induced corticosterone levels (the primary avian glucocorticoid). Briefly, chicks were bled within 3 minutes of handling to measure baseline corticosterone levels, and then placed in a cloth bag for 30 minutes and then bled again to measure stress-induced corticosterone levels. Blood samples were stored on ice for less than 5 hours, separated, and stored at -80˚C until further analysis. A

whole blood sample was also taken at 10 days post-hatch, and snap-frozen on dry ice for later telomerase activity analysis. To collect tissues, chicks were euthanized and tissues (bone marrow, liver, and pectoralis muscle) were dissected, aliquoted, and snap frozen on dry ice. Bone marrow was collected from both femurs and stored in 60 µL of PBS. All tissues were stored at -80˚C.

Measuring Telomerase Levels

Telomerase levels were measured using a TRAP assay and TERT gene expression. The TRAP assay measures the amount of active telomerase enzyme within a tissue. TERT gene expression measures the amount of TERT RNA within a tissue, where TERT is the reverse transcriptase protein subunit that partially makes up the telomerase enzyme and has been previously used as a proxy for telomerase activity.

TRAP Assay

To measure telomerase enzymatic activity, we used the TRAPeze RT Telomerase Detection Kit (Sigma-Aldrich, S7710). Tissues were extracted following the kit protocol. Briefly, about 10 mg of tissue was placed in 100 µL of CHAPS lysis buffer and ground to a consistent substrate. Samples were then incubated on ice for 30 minutes and centrifuged at 12,000 x g for 20 minutes. The supernatant was pipetted off and placed in a new Eppendorf tube. Extracted samples were not thawed more than 10 times to ensure the protein was not degraded. To determine the concentration of protein within each sample, a Bradford assay was run on all extracted samples. Each sample was then diluted to 500 ng/µL using CHAPS lysis buffer. Quantitative real-time PCR (qPCR) (Stratagene Mx 3000P) was run in order to measure telomerase activity. Wells contained 17.6 µL of sterile water, 5 µL of TRAPeze RT Reaction Mix, 0.4 µL of Taq polymerase (Titanium Taq, Takara), and 2 µL of the diluted sample. A

standard curve of 2,408,800, 240,880, 24,080, and 2,400 were generated using serial dilutions of the kit's oligonucleotide control template, which was run in triplicate. Each plate included a positive control (telomerase-positive cells diluted in CHAPS lysis buffer) and a non-template control (sterile water). These controls and all samples were run in duplicate. qPCR thermal profile was 30 minutes at 30˚C, 2 minutes at 95˚C, and 45 cycles of 15 seconds at 94˚C, 60 seconds at 59˚C, and 30 seconds at 45˚C. Following the protocol, telomerase activity was calculated by plotting the log scale of the standard curves copy number against the corresponding Ct value. The mean Ct value for each sample was then fitted to the standard curve linear regression plot, and the copy number for each sample was found. This is the copy number added by active telomerase in each sample.

TERT Gene Expression

The RNAzol RT method (Molecular Research Center) was used to extract tissues (pectoralis muscle and liver), snap frozen on dry ice, and stored at -80˚C. Using the qScript® cDNA Synthesis Kit (Quanta bio), mRNA was reverse transcribed into cDNA. mRNA gene expression levels were calculated using qPCR. cDNA standards for qPCR were prepared by reverse transcription PCR and cloned into the pGEM-T Easy Vector (Promega). Gene specific primers were designed by utilizing the house sparrow genome (Accession # PRJNA255814) and the PrimerQuest tool from Integrated DNA Technologies. All qPCR primer sets were validated by melt curve analysis and gel electrophoresis to confirm a single amplicon of the correct size. The house keeping gene was Beta actin and was also validated using the reference genome. The TERT thermal profile was 10 minutes at 95˚C and 45 cycles of 15 seconds at 95˚C, 30 seconds at 58˚C, and 30 seconds at 72˚C. The TERT standard curve was a serial dilution of 5,000,000, 500,000, 50,000, 5,000, and 500 copies. The Beta actin thermal profile was 10 minutes at 95˚C,

and 40 cycles of 15 seconds at 95˚C, 30 seconds at 58˚C, and 30 seconds at 72˚C. The Beta actin standard curve was a serial dilution of 3,000,000, 600,000, 120,000, 24,000, 4,800, and 960 copies. TERT and Beta actin reactions were run on separate plates, and all samples fell within the boundaries of their respective standard curves. Well reactions used 12.5 µL PerfeCTa SYBR® Green SuperMix (Quanta bio), 0.5 µL of the TERT or Beta actin primer, 6 µL of RNA-free sterile water, and 6 µL of 1:3 diluted sample, for a total 25 µL reaction. A water sample was included on each plate as a negative control. A golden sample was included on each plate and consisted of a pool of 4 individuals. For both genes, the standard and golden sample was run in triplicate, and samples were run in duplicate.

From the qPCR reaction, Ct values are calculated as the number of PCR cycles were needed to accumulate enough fluorescent signal to cross a specified threshold. TERT gene expression was reported as the T/S ratio and calculated by taking the samples average Ct value and determining the fold change using the $2^{\Delta\Delta Ct}$ formula where $\Delta\Delta Ct = (C_t^{TERT} - C_t^{B\ Actin})$sample $- (C_t^{TERT} - C_t^{B\ Actin})$reference [30].

Measuring Stress Hormone Levels

Corticosterone levels were measured using the Corticosterone ELISA Kit (Enzo Life Sciences). Plasma samples were extracted with 1 ml of diethyl ether, snap frozen in a methanol ice bath, and the supernatant was transferred to a new tube. This was repeated to ensure all supernatant was extracted. Samples were then blown down under nitrogen gas until dry, and then resuspended in 300 µL of assay buffer. All samples were run on a 96 well plate according to the manufacturers' instructions. The standard curve of 20,000, 4,000, 1,000, 250, 62.5, and 15.63 pg/ml was run on the plate. Total activity (TA), non-specific binding (NSB), maximum binding (B0), and a blank was included on the plate as controls. The standard and controls were run in

triplicate and the samples were run in duplicate. Plates were run on a spectrophotometer and read at 405 nm and 570 nm using the Ascent Software.

Measuring Relative Telomere Length

Blood and tissues (pectoralis muscle and liver) were extracted for DNA using the Machery-Nagel Nucleospin® Blood and Tissue kits. To determine DNA purity and concentration, samples were analyzed using a NanoDrop 8000 (Thermo Scientific®). Samples were diluted to 4 ng per 1 µL. Following previously established protocols [5,29], relative telomere length was measured using qPCR. GAPDH was used as the housekeeping gene with a qPCR thermal profile was 10 minutes at 95˚C, and 40 cycles of 30 seconds at 95˚C and 30 seconds 60˚C. The telomere thermal profile for qPCR was 10 minutes at 95˚C, and 27 cycles of 15 seconds at 95˚C, 30 seconds at 58˚C, and 30 seconds at 72˚C. Reaction wells contained 6 µL of sterile water, 12.5 of PerfeCTa SYBR® Green SuperMix (Quanta bio), and 0.25 of each forward and reverse primers. A serial dilution of a reference sample was used to produce a standard curve of 40, 20, 10, 5, and 2.5 ng. A tissue specific reference sample and the standard curve was run in triplicate on each plate. A sterile water sample was included on each plate as a negative control. All samples and the negative control were run in duplicate.

Telomere length was determined by calculating the Ct value were Ct is the number of PCR cycles needed to accumulate enough fluorescent signal to cross a specified threshold. The average Ct value was calculated for each sample and was used to determine the T/S ratio according to the $2^{\Delta\Delta Ct}$ formula where $\Delta\Delta Ct = (C_t^{Telo} - C_t^{GAPDH})sample - (C_t^{Telo} - C_t^{GAPDH})reference$ [30].

Determining Molecular Sex

Due to nestlings sex not being able to be distinguished at this age, genetic sex is needed to determine this. DNA extracts (see above) were diluted to 10 ng/μL, and then amplified using polymerase chain reaction (PCR). The primers used were P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'-CTCCCAAGGATGAGRAAYTG-3'). Samples were then run using gel electrophoresis and visualized using a FluorChem FC2 imaging system. Females show two bands due to their heterogenous karyotype (ZW), and males show one band due to their homogeneous karyotype (ZZ).

Statistical Analysis

Telomerase activity is reported as copy number as described in the TRAP Assay methods section. Relative telomere length at 10 days old is reported as T/S ratio as described above. The change in telomere length was calculated as the final length (10 days post-hatch) minus the initial length (2 days post-hatch) adjusted for the regression to the mean (RTM). The RTM equation is

$$RTM = -1\left(p(X_1 - \bar{X}_1) - (X_2 - \bar{X}_2)\right) \ where \ \rho = \frac{2rs_1s_2}{s_1^2 + s_2^2}$$

where X_1 is T/S ratio at 2 days old, $\bar{X}_1$ is the mean of 2 day old T/S ratios, X_2 is T/S ratio at 10 days old, $\bar{X}_2$ is the mean of 10 day old T/S ratios, r is the correlation coefficient between 10 day old T/S ratios and 2 day old T/S ratios, s is standard deviation, and s^2 is variance.

One significant outlier was removed from the dataset because the pectoralis TERT gene expression value was greater than two standard deviations away from the mean. General linear mixed effects models were used to determine whether the treatment and telomerase activity had an effect on telomere length. Ordinal date and sex were not significant in any of the models and were removed from further analysis. Nest was included in all models as a random effect to

control for the fact that multiple chicks were sampled from the nest. To test potential effects of the treatment on growth, telomerase levels, and telomere length, all models included treatment as a fixed effect. In models where telomere length was the dependent variable, telomerase activity of the related tissue was included as a covariate. To test how the treatment and telomerase activity affected telomere length, a treatment by telomerase activity interaction was included in models where telomere length was the dependent variable. In models for pectoralis muscle and liver telomeres, and change in telomere length models, the interaction between treatment and telomerase activity was not significant and was removed from the analysis.

Corticosterone levels were natural log transformed to improve normality. To assess whether the stress treatment influenced corticosterone levels, we used a linear mixed effect model that included natural log transformed corticosterone levels as the dependent variable, and treatment and the time point (baseline or stress-induced) as fixed effects. All statistical analyses were performed in RStudio (R Version 3.4.3 with packages lme4 [35], lmerTest [36], ggpubr, ggplot2). Models are presented with the results in Appendix B.

Results

Effects of the Experimental Treatment

Chicks in the stressed treatment group had significantly higher corticosterone levels ($F_{1,25.8} = 7.26$; $p = 0.012$) (Fig. 5A) and reduced body mass at the end of the growth period ($F_{1,7.32} = 7.44$; $p = 0.028$) (Fig. 5B), but there were no significant effects of the treatment on any of the other growth measures (Appendix B). There was no significant effect of the treatment on telomeres in any of the tissues (blood, pectoralis muscle, or liver) (Appendix B). Telomerase was not detectable in whole blood in any of the samples from either treatment group. There was no significant effect of the treatment on telomerase activity in the liver ($F_{1,15} = 0.38$; $p = 0.548$) or

bone marrow ($F_{1,16} = 0.09$; $p = 0.765$). However, telomerase activity in stressed chicks tended to be elevated in the pectoralis muscle ($F_{1,200} = 3.78$; $p = 0.053$) relative to controls (Fig. 6).

There was no significant effect of the treatment on TERT gene expression in pectoralis muscle ($F_{1,7.76} = 3.22$; $p = 0.112$) or liver ($F_{1,14} = 1.04$; $p = 0.325$). TERT gene expression was also not significantly correlated with telomerase activity in either tissue (Pec: $p = 0.089$; Liver: $p = 0.63$). Telomerase activity is the amount of active telomerase in a tissue, whereas TERT gene expression is the amount of TERT, a subunit of telomerase, RNA being expressed. Due to this lack of relationship, TERT gene expression was not related to the amount of active telomerase within a tissue and therefore it does not seem to be a viable alternative to measure telomerase, and so was removed from further analysis.

The Relationship Between Telomerase Activity and Telomeres

At 10 days post-hatching, bone marrow telomerase was significantly positively related to blood telomere length ($F_{1,4.95} = 21.5$; $p = 0.006$), where birds with more active telomerase in their bone marrow had longer blood telomeres (Fig. 7A). There was also a near significant treatment x bone marrow telomerase activity interaction effect on 10 day old telomere length ($F_{1,6.98} = 5.22$; $p = 0.056$), where stress birds tended to have a more positive relationship between telomerase activity and telomere length at 10 days post-hatching than controls (Fig. 7B). The change in blood telomere length (corrected for RTM) had a significant positive relationship with bone marrow telomerase activity ($F_{1,5.78} = 9.51$; $p = 0.023$), where chicks with more telomerase activity in their bone marrow had less telomere loss in blood telomeres over time (Fig. 8). In contrast, liver telomerase activity and telomere length were significantly negatively related to one another ($F_{1,2.41} = 20.2$; $p = 0.032$), where individuals with greater telomerase activity in the liver had shorter liver telomeres (Fig. 9). There was no significant interaction between treatment

and liver telomerase activity on liver telomere lengths. Pectoralis muscle telomerase activity and telomere length were not significantly related to one another ($F_{1,10} = 3.22$; $p = 0.103$), and there was no significant treatment x pectoralis muscle telomerase activity interaction on pectoralis telomere length. Telomerase activity was not correlated between the different tissues of liver, pectoralis muscle, and bone marrow ($p = >0.1$), and telomere length was also not correlated between the different tissues of liver, pectoralis muscle, and blood ($p = >0.2$).

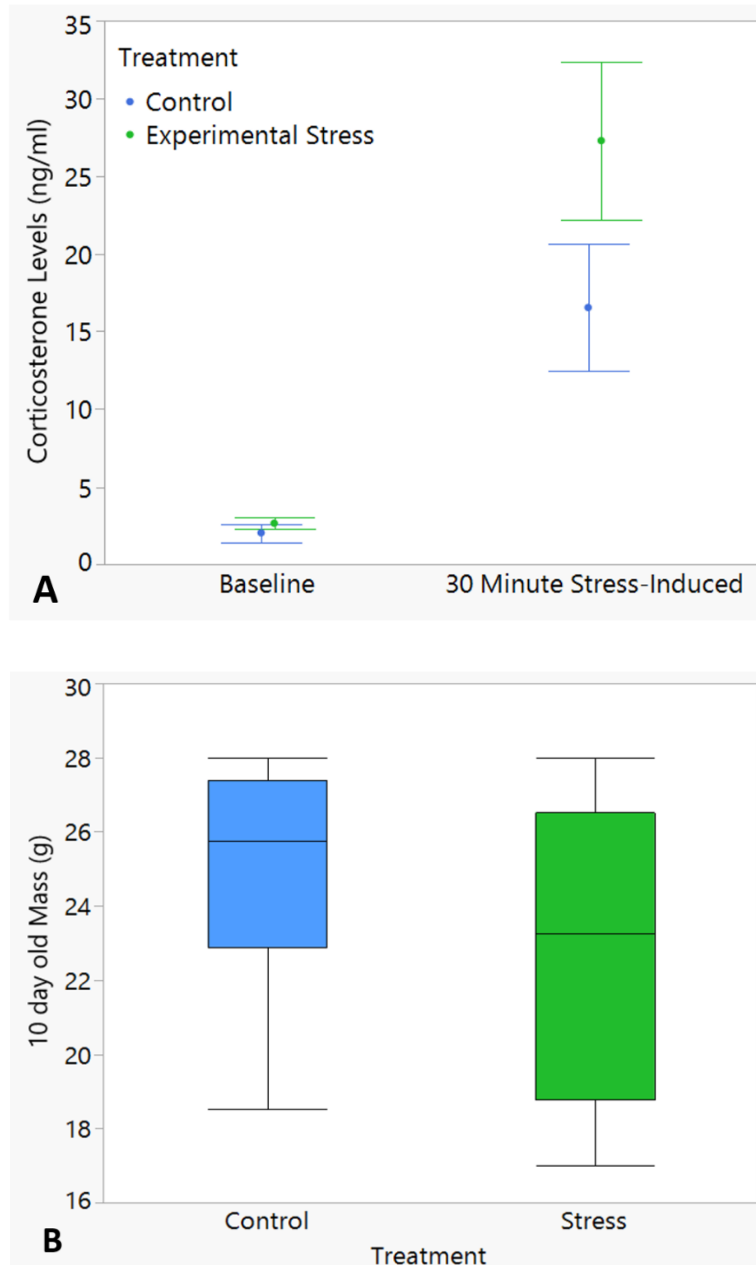


Figure 5: (A) The influence of treatment on baseline and 30 minute stress-induced corticosterone levels in 10 day old house sparrow chicks. Corticosterone levels were significantly higher in the chicks in the stress treatment group relative to controls ($F_{1,25.8} = 7.26$; $p = 0.012$). (B) The relationship between treatment and house sparrow chick mass at 10 days old. Stressed chicks weighed significantly less than controls at the end of post-natal growth ($F_{1,7.32} = 7.44$; $p = 0.028$). Sample size for the experimental stress group is $n = 10$, and the control group is $n = 8$.

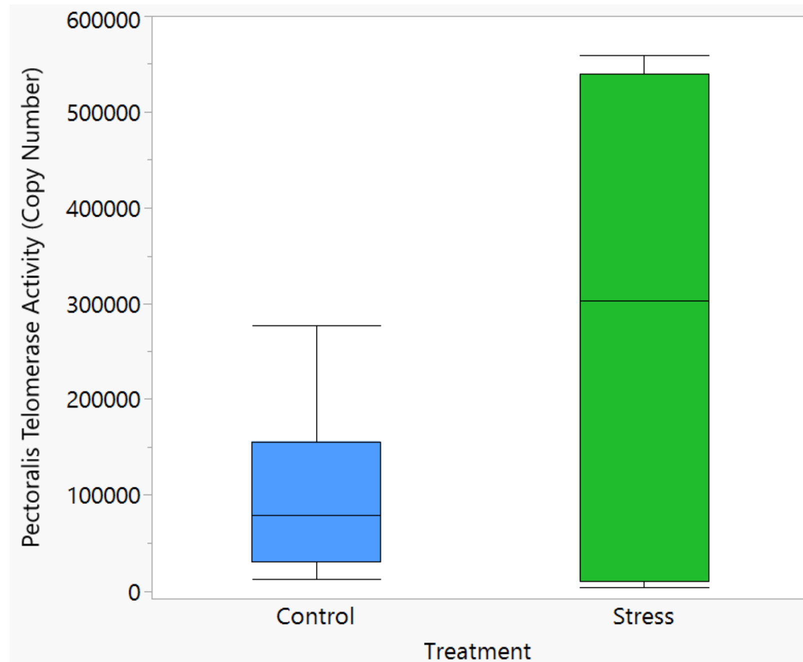
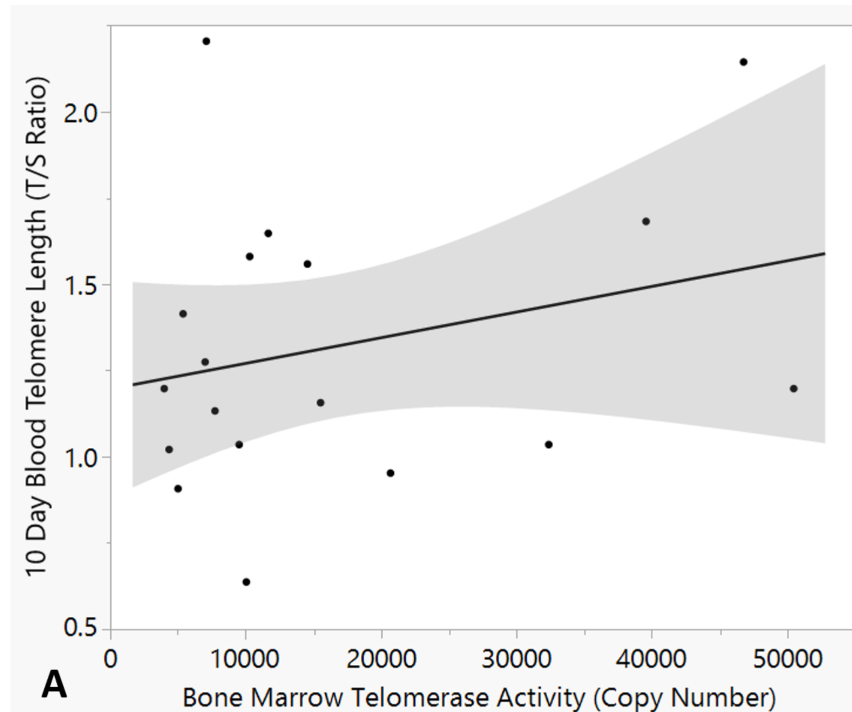


Figure 6: The relationship between treatment and pectoralis muscle telomerase activity in 10 day old house sparrow chicks. Pectoralis muscle telomerase activity tended to be higher in stressed chicks ($F_{1,200} = 3.78$; $p = 0.053$) than controls. Sample size for the stress treatment is n = 7, and control treatment is n = 8. Telomerase activity is reported as the amount of copies added to an oligonucleotide by active telomerase within the tissue in 30 minutes.



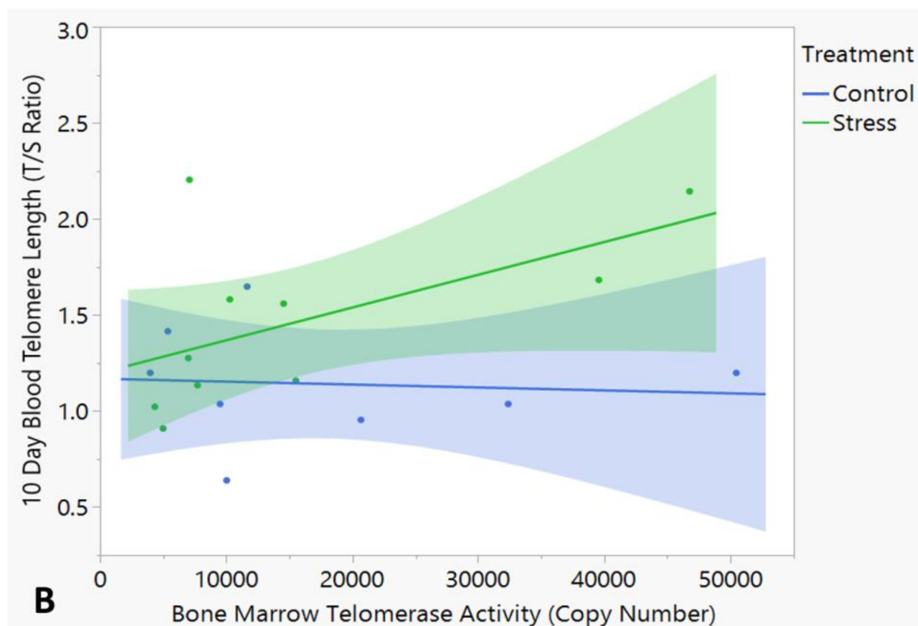


Figure 7: (A) The relationship between bone marrow telomerase activity and blood telomere length in 10 day old house sparrow chicks. Bone marrow telomerase activity and telomere length are significantly positively related to one another ($F_{1,4.95} = 21.5$; $p = 0.006$). (B) This relationship also tended to be more positive in stressed chicks (green) than controls (blue) ($F_{1,6.98} = 5.22$; $p = 0.056$). Sample size for the experimental stress group is $n = 10$, and the control group is $n = 8$. Telomere length is reported as T/S ratio which is calculated using $2^{\Delta\Delta Ct}$ formula (see methods section). Telomerase activity is reported as the amount of copies added to an oligonucleotide by active telomerase within the tissue in 30 minutes.

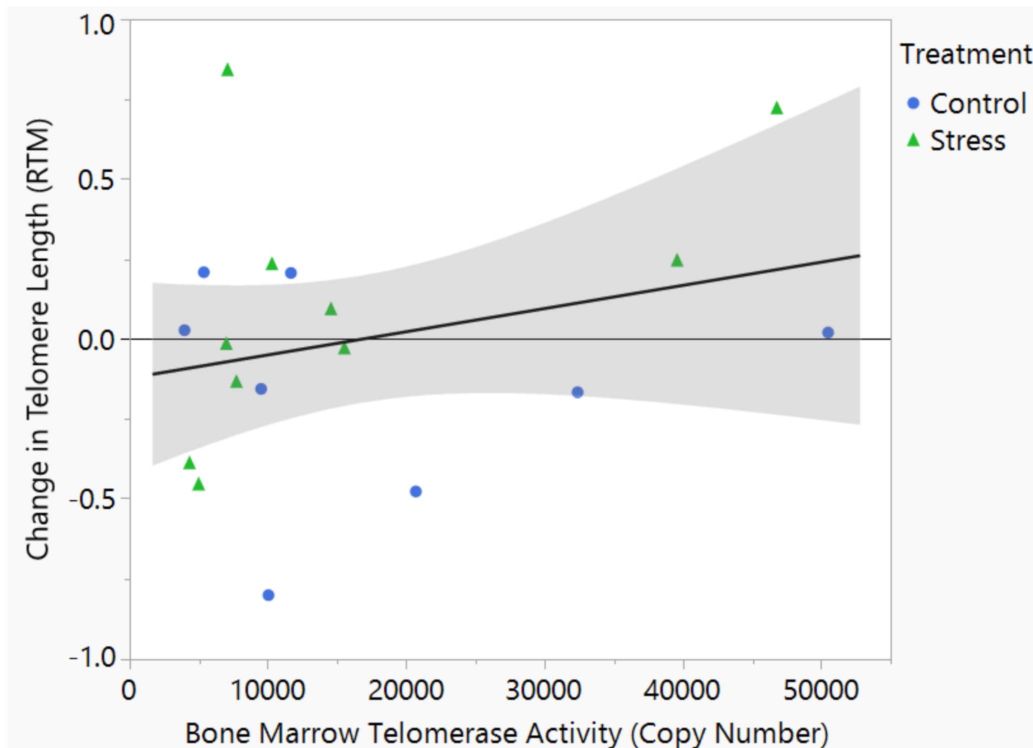


Figure 8: The relationship between bone marrow telomerase activity and the change in telomere length (corrected for RTM) in blood in house sparrow chicks. Chicks with greater bone marrow telomerase activity experienced significantly less telomere loss in blood ($F_{1,5.78} = 9.51$; $p = 0.023$). The line that starts at zero on the Y-axis represents no change in telomere length across the growth period. Sample size for stress treatment is $n = 10$, and control group is $n = 8$. Telomerase activity is reported as the amount of copies added to an oligonucleotide by active telomerase within the tissue in 30 minutes. Change in telomere length is denoted by 10 days post-hatch telomere length minus 2 days post-hatch telomere length, corrected to the regression to the mean (RTM).

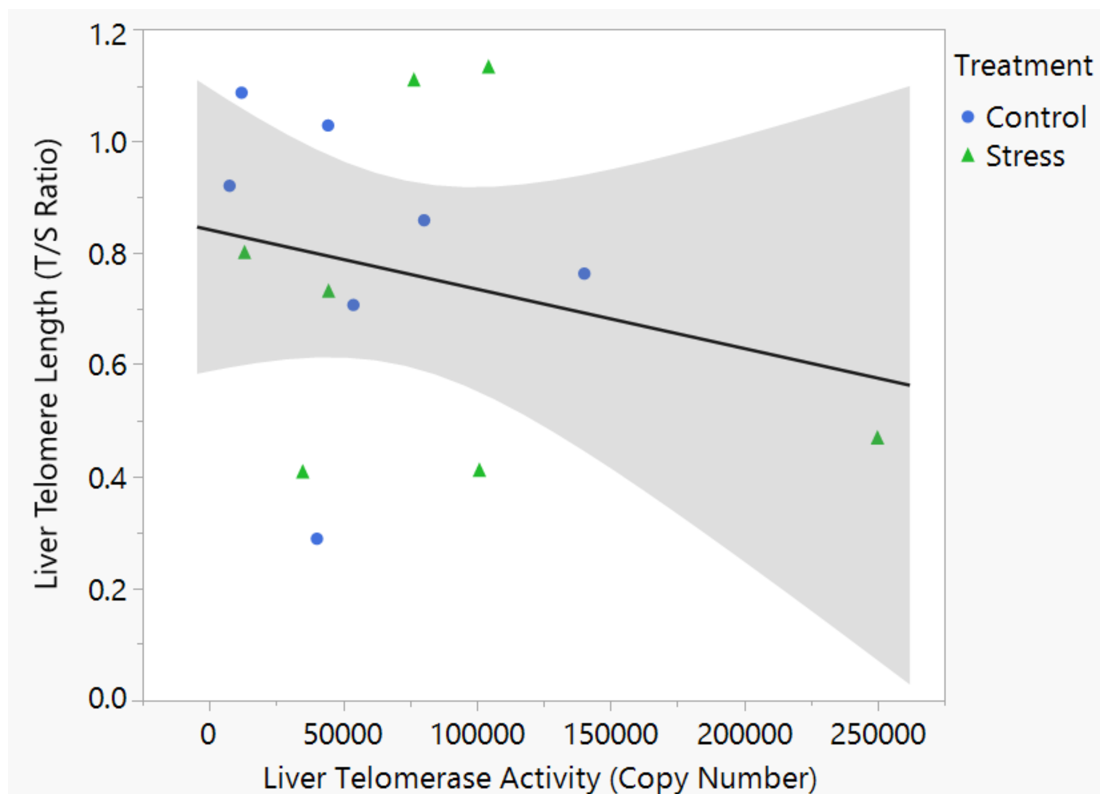


Figure 9: The relationship between telomere length and telomerase activity in the liver in 10 day old house sparrow chicks. Liver telomerase activity had a significant negative relationship with liver telomere length ($F_{1,2.41} = 20.2$; $p = 0.032$), where individuals with lower telomerase activity had longer telomeres in their liver. Sample size for stress treatment group is $n = 7$, and control group is $n = 7$. Telomere length is reported as T/S ratio which is calculated using $2^{\Delta\Delta Ct}$ formula (see methods section). Telomerase activity is reported as the amount of copies added to an oligonucleotide by active telomerase within the tissue in 30 minutes.

Discussion

Exposure to stress during early life often has negative effects on telomeres, but this can vary considerably among individuals and the mechanisms that contribute to variation in resilience are not well understood. Here we tested if telomerase is one mechanism that contributes to variation in the effects of stress on telomeres during development. Our experimental treatment was effective at increasing developmental stress, as stressed chicks had significantly higher corticosterone levels and lower body mass at the end of the treatment than controls. However, despite this, there were no significant effects of the stress treatment on

telomeres in any of the tissues measured in this study. With the exception of blood, telomerase was active during post-natal development in all of the tissues we examined. In both treatment groups, chicks with higher bone marrow telomerase activity experienced less telomere loss and had longer telomeres at the end of post-natal growth. Interestingly, we also found that pectoralis muscle telomerase activity tended to be higher in stressed chicks than controls. Taken together, these results suggest that telomerase activity is positively associated with telomere length and may be upregulated in response to stressors, but that these effects can vary considerably among tissues. This is consistent with previous literature where adult mice and humans upregulated telomerase in response to stress[21,22], and telomerase has been shown to vary considerably across tissues[37].

Telomerase was active during post-natal development and this is consistent in other avian species[37,44]. Yet the relationship between telomerase and telomeres during early life has not been previously studied. We found that chicks with more telomerase activity in their bone marrow gained more telomeres in their blood over the post-natal growth period. This is why it is important to look at telomerase activity relative to telomere length over time in order to fully characterize the effects on longevity as it may regulate shortening and longevity[45]. We also saw a positive relationship between bone marrow telomerase and blood telomeres consistent with our predictions[1]. However, this same positive relationship was not observed in the liver and muscle. One reason for this may be that we were only able to collect a single terminal sample from these tissues and it may only represent a 'snapshot' of the organisms' physiological reactions to stress, rather than the overall effect. Because telomerase is not detected in blood in post-natal developing chicks, we are currently unable to track telomerase activity levels across the growth period without terminal sampling. To try to avoid terminal sampling, previous studies have used

TERT gene expression, a subunit of telomerase, as a proxy of telomerase activity. However we found no relationship between these measures in liver or pectoralis muscle in house sparrow chicks. Due to the lack of relationship, TERT gene expression is not connected to active telomerase levels found within cells during post-natal development and may not be a good alternative, at least in this species. Future studies should investigate techniques of measuring telomerase activity and telomeres over to better characterize this dynamic process.

Our findings also showed a tendency for telomerase to be upregulated in response to stress in the pectoralis muscle, and this is consistent with previous studies. Adult mice have been shown to upregulate telomerase activity in white blood cells in response to stress[21], and this upregulation could be a cellular mechanism to buffer against the effects of stress on telomeres. However, an upregulation of telomerase in response to stress was not observed in any other tissue. Due to this increase in activity, telomeres in the pectoralis muscle may be more protected against stress and therefore have an extended cellular lifespan. Organisms may differentially protect tissues in response to stress and muscle may be relatively more buffered because it is less proliferative and/or critical for survival, especially in avian species where flight is paramount. Understanding how the impact of stress on telomerase expression varies among tissues and the consequences for telomere dynamics is an important area for future research.

How stress affects telomerase activity during post-natal development has not been previously characterized, and has only been investigated in adult mammals where in white blood cells telomerase activity was higher in stressed individuals [21,22]. Due to the different physiological functions of white blood cells versus the tissues we measured, the mechanisms behind protecting cellular longevity could be very different. However, how age of the organism, such as adulthood or post-natal development, affects these mechanisms as well as telomerase

levels are still poorly understood. Our findings show that even during this early life period, telomerase activity is not only active, but may also upregulated in response to environmental stress in order to protect telomeres in certain tissues. But how telomerase can influence lifetime stress on telomere maintenance is still not known. Future research may be able to find a measure of telomerase that represents the whole body levels and can be sampled without terminal procedures. Telomerase activity may be one of the important mechanisms that contribute to the variation in response to stress and is a critical piece to understanding how organisms are able to combat environmental challenges.

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

Major Findings

In this thesis, I conducted a series of experimental studies to answer the following questions. 1) How does experimental manipulation of telomerase during development impact telomeres?, and 2) To what degree does telomerase vary in response to stress and impact telomeres during development?

In chapter 2, I experimentally exposed free-living house sparrow chicks to TA-65, a telomerase activator, during post-natal development and examined the effects on telomere length and loss, and growth of keratin-based structures. Experimental chicks received a daily oral dose of TA-65 from 2 to 10 days old, and control chicks received a water dose. Contrary to expectation, experimental chicks experienced more telomere loss relative to controls and the growth of keratin-based structures was reduced in TA-65 chicks in the second year of the study. These results are quite novel, especially because previous studies on adult organisms had seen an increase in telomeres and keratin-based structural growth with supplementation of TA-65[11–13]. There are a variety of reasons why the results differ with respect to life-stage. TA-65 may be inadvertently suppressing endogenous telomerase activity during post-natal development, and this could be causing shorter telomeres. The negative effect of TA-65 on keratin-based structure growth could be impacting cellular division and proliferation capacity during this time period of exponential growth. These results demonstrate that the effects of TA-65 on telomeres and the growth of keratin-based structure growth differs across life stages, and provides novel insight into our ability to manipulate telomeres during post-natal growth.

In chapter 3, I chronically stressed house sparrow chicks during post-natal growth and examined the effects on telomerase and telomeres. Between days 2 to 10 post-hatching, chicks in

the experimental stress treatment were removed from the nest and exposed to a standardized handling stressor for 30 minutes each day, whereas control chicks remained undisturbed in the nest. To assess the effects of the treatment on stress hormones, telomerase, and telomeres, at 10 days post-hatching, chicks were exposed to a standardized stress series to measure their baseline and stress-induced corticosterone levels (the primary avian glucocorticoid stress hormone), euthanized, and several tissues were collected liver, pectoralis muscle, and bone marrow. Interestingly, although the chicks exposed to the stress treatment had elevated corticosterone levels and reduced body mass at the end of post-natal growth there were no significant effects of the treatment on telomere length. However, stressed chicks tended to have higher telomerase activity in their pectoralis muscle. Stress commonly impacts telomeres, but an upregulation of telomerase activity may buffer the effects of stress on telomeres. Chicks that had higher telomerase activity in their bone marrow during the post-natal growth period also experienced less telomere loss. Our results demonstrate that telomerase is active during post-natal development, and that upregulation in response to stress may protect telomeres in some tissues.

Future Directions

These studies enhance our understanding of how telomerase influences telomere dynamics during post-natal development under a variety of conditions. Future research should consider understanding the mechanisms behind individual variation in telomerase and the associated pathways, as this would improve our understanding about the high amount of telomere length variation between individuals. How the levels of telomerase activity change throughout post-natal growth is still unknown for most species other than humans, and this information could be a key mechanism of individual variation in telomere dynamics as it changes across this time period. Studies have shown that early life telomere length is predictive of

lifespan[5] yet we still do not know how telomerase impacts telomere lengths during this critical life stage.

To understand how telomerase impacts telomeres across development, it will be essential to sample telomerase and telomeres at multiple time points. Longitudinal studies within individuals would be ideal, but a significant barrier to these types of studies is that measuring telomerase in most tissues requires terminal sampling. I did not detect any telomerase activity in the blood. However, one measure of telomerase activity that has been previously used in blood is TERT gene expression, which is a subunit of telomerase. Yet my findings indicate that telomerase activity and TERT gene expression are not significantly related within tissues, and more research is needed to determine whether this is a possible method for measuring longitudinal changes in telomerase activity in blood in other species and life stages.

In addition, future research should continue to explore ways to manipulate telomere length through changes in telomerase levels to better understand the relationship between early life telomeres, longevity, and other life-history traits. Manipulating telomerase through the use of TA-65 may make it possible to experimentally investigate the causal links between telomerase and telomeres. However, the pathways that TA-65 acts through are not well characterized. A better understanding of the mechanistic pathways in which TA-65 influences telomerase during development could also inform new ways to experimentally manipulate telomere length.

The research presented in this thesis is only one step in many to understanding the factors that contribute to the variation in telomeres and aging rates. With this novel information, future studies should seek to understand the mechanisms of cellular aging during early life and the ability to mitigate the detrimental effects of stress during early life on aging.

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APPENDIX A: A SUMMARY OF THE COMPONENTS AND RESULTS OF THE GENERAL LINEAR MIXED EFFECTS MODELS USED TO RUN THE EXPERIMENTAL RESULTS IN RSTUDIO USING THE LME4 PACKAGE. RESULTS FROM 2016 AND 2017 DATA (A), AS WELL AS SEPARATE MODELS THAT ANALYZED JUST 2016 (B) AND 2017 (C) DATA INDIVIDUALLY

A	**2016 & 2017 Data:**				
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
2 day old Telomere Length					
	Treatment	-0.023 (0.091)	1, 20.7	0.06	0.804
	Year	-0.067 (0.109)	1, 14.9	0.37	0.549
	Age	0.120 (0.056)	1, 104.9	4.53	**0.035**
	Sex	-0.062 (0.069)	1, 113.7	0.81	0.369
	Clutch Size	-0.079 (0.042)	1, 40.8	3.5	0.068
	Ordinal Date	0.002 (0.002)	1, 23.8	1.03	0.319
2 day old Mass					
	Treatment	-0.333 (0.398)	1, 24.2	0.70	0.410
	Year	0.229 (0.323)	1, 78.5	0.50	0.480
	Age	1.800 (0.143)	1, 116.9	159.1	**<0.0001**
	Sex	0.045 (0.162)	1, 110.3	0.08	0.781
	Clutch Size	-0.156 (0.135)	1, 100.8	1.33	0.251
	Ordinal Date	0.008 (0.010)	1, 27.2	0.66	0.424
10 day old Telomere Length					
	Treatment	0.033 (0.104)	1, 22.7	1.56	0.223
	Year	0.142 (0.128)	1, 10.7	0.09	0.770
	Treatment*Year	-0.221 (0.134)	1, 35.9	2.76	0.105
	Age	-0.021 (0.062)	1, 89.7	0.11	0.737
	Sex	-0.093 (0.055)	1, 88.7	2.82	0.097
	Clutch Size	-0.031 (0.030)	1, 35.4	1.07	0.308
	Ordinal Date	0.001 (0.002)	1, 30.5	0.41	0.528
Change in Telomere (RTM)					
	Treatment	0.086 (0.101)	1, 17.2	2.11	0.164
	Year	0.229 (0.131)	1, 10.2	0.34	0.573
	Treatment*Year	-0.289 (0.132)	1, 26.9	4.76	**0.038**
	Age	-0.045 (0.062)	1, 78.8	0.53	0.468
	Sex	-0.102 (0.056)	1, 78.3	3.33	0.072
	Clutch Size	-0.055 (0.031)	1, 31.1	3.24	0.081
	Ordinal Date	0.003 (0.002)	1, 27.0	2.31	0.140

A		2016 & 2017 Data:			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
Rectrices					
	Treatment	1.602 (1.215)	1, 142.8	1.74	0.189
	Age	3.101 (0.127)	1, 325.0	684.8	**<0.0001**
	Treatment*Age	-0.316 (0.125)	1, 203.2	6.36	**0.012**
	Year	0.319 (0.648)	1, 57.1	0.24	0.624
	Mass	0.378 (0.052)	1, 307.4	53.3	**<0.0001**
	Sex	0.317 (0.397)	1, 95.9	0.639	0.426
	Clutch Size	-0.298 (0.282)	1, 68.8	1.12	0.294
	Ordinal Date	0.029 (0.019)	1, 25.5	2.49	0.127
Culmen					
	Treatment	0.129 (0.100)	1, 57.1	1.65	0.204
	Age	0.237 (0.017)	1, 474.6	196.4	**<0.0001**
	Treatment*Age	-0.022 (0.009)	1, 383.8	4.8	**0.029**
	Year	-0.297 (0.075)	1, 69.3	15.8	**0.0002**
	Mass	0.089 (0.006)	1, 468.9	193.9	**<0.0001**
	Sex	0.062 (0.044)	1, 122.8	1.93	0.167
	Clutch Size	0.066 (0.032)	1, 86.3	4.17	**0.044**
	Ordinal Date	0.007 (0.002)	1, 29.1	11.5	**0.002**
Wing Chord					
	Treatment	0.487 (0.886)	1, 107.4	0.30	0.583
	Age	2.759 (0.172)	1, 457.3	275.5	**< 0.0001**
	Treatment*Age	-0.201 (0.108)	1, 460.7	3.47	0.063
	Year	0.018 (0.584)	1, 62.0	0.001	0.975
	Mass	0.715 (0.063)	1, 450.3	128.4	**< 0.0001**
	Sex	-0.283 (0.371)	1, 448.5	0.58	0.446
	Clutch Size	0.224 (0.252)	1. 78.2	0.78	0.38
	Ordinal Date	0.018 (0.016)	1, 27.8	1.29	0.264
Pin Feathers					
	Treatment	1.532 (1.413)	1, 129.9	1.17	0.280
	Age	2.095 (0.127)	1, 272.6	325.3	**<0.0001**
	Treatment*Age	-0.263 (0.139)	1, 162.1	3.58	0.06
	Year	-0.156 (0.665)	1, 59.9	0.05	0.816
	Mass	0.044 (0.052)	1, 254.6	0.69	0.406
	Sex	-0.033 (0.381)	1, 80.0	0.007	0.931
	Clutch Size	0.052 (0.283)	1, 73.7	0.03	0.855
	Ordinal Date	0.022 (0.021)	1, 23.3	1.15	0.293

A		**2016 & 2017 Data:**			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
10 day old Mass					
	Treatment	-0.533 (0.692)	1, 21.1	0.59	0.449
	Age	2.412 (0.532)	1, 94.9	20.5	**<0.0001**
	Year	2.096 (0.695)	1, 57.6	9.10	**0.004**
	Sex	-0.648 (0.515)	1, 108.5	1.58	0.211
	Clutch Size	-0.543 (0.322)	1, 58.1	2.83	0.098
	Ordinal Date	0.069 (0.018)	1, 22.9	14.6	**0.0009**

B		**2016 Data:**			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
10 day old Telomere Length					
	Treatment	0.007 (0.096)	1, 23.3	0.01	0.940
	Age	-0.316 (0.096)	1, 17.5	10.8	**0.004**
	Sex	0.158 (0.097)	1, 23.5	2.65	0.117
	Clutch Size	-0.078 (0.047)	1, 23.1	2.76	0.110
	Ordinal Date	0.006 (0.002)	1, 1.71	8.99	0.115
Change in Telomeres (RTM)					
	Treatment	0.020 (0.103)	1, 4.55	0.04	0.851
	Age	-0.283 (0.097)	1, 17.5	8.47	**0.009**
	Sex	0.148 (0.098)	1, 23.7	2.25	0.147
	Clutch Size	-0.051 (0.050)	1, 4.27	1.04	0.362
	Ordinal Date	0.005 (0.002)	1, 17	5.24	0.232
Rectrices					
	Treatment	3.516 (1.938)	1, 42.2	3.29	0.077
	Age	2.633 (0.190)	1, 72.9	215.9	**<0.0001**
	Treatment*Age	-0.615 (0.206)	1, 52.7	8.95	**0.004**
	Mass	0.564 (0.069)	1, 48.2	67.1	**<0.0001**
	Sex	0.437 (0.541)	1, 20.7	0.65	0.428
	Clutch Size	-0.212 (0.502)	1, 4.45	0.18	0.692
	Ordinal Date	0.022 (0.023)	1, 4.25	0.93	0.386
Culmen					
	Treatment	-0.161 (0.165)	1, 20.5	0.95	0.341
	Age	0.228 (0.029)	1, 74.0	76.1	**<0.0001**
	Treatment*Age	0.018 (0.019)	1, 93.8	0.88	0.348
	Mass	0.092 (0.011)	1, 65.7	64.1	**<0.0001**
	Sex	0.169 (0.106)	1, 28.6	2.56	0.121
	Clutch Size	0.134 (0.058)	1, 4.03	5.39	0.080
	Ordinal Date	-0.002 (0.002)	1, 3.01	0.76	0.448

B		**2016 Data:**			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
Wing Chord					
	Treatment	1.631 (1.518)	1, 25.6	1.15	0.293
	Age	2.468 (0.259)	1, 81.7	98.8	**<0.0001**
	Treatment*Age	-0.323 (0.183)	1, 112.8	3.13	0.079
	Mass	0.844 (0.096)	1, 73.7	77.8	**<0.0001**
	Sex	-0.393 (0.705)	1, 115.9	0.31	0.578
	Clutch Size	-0.036 (0.497)	1, 4.63	0.005	0.945
	Ordinal Date	-0.002 (0.022)	1, 3.79	0.008	0.934
Pin Feathers					
	Treatment	3.189 (2.932)	1, 58.5	1.18	0.281
	Age	1.766 (0.253)	1, 64.9	61.5	**<0.0001**
	Treatment*Age	-0.449 (0.311)	1, 60.5	2.09	0.153
	Mass	0.236 (0.075)	1, 64.2	9.79	0.003
	Sex	0.145 (0.548)	1, 63.7	0.07	0.792
	Clutch Size	0.017 (0.593)	1, 5.00	0.001	0.978
	Ordinal Date	0.013 (0.028)	1, 5.13	0.21	0.666

C		**2017 Data:**			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
10 day old Telomere Length					
	Treatment	-0.221 (0.076)	1, 53.8	8.53	**0.005**
	Age	0.077 (0.072)	1, 62.3	1.14	0.289
	Sex	-0.097 (0.065)	1, 61.7	2.25	0.139
	Clutch Size	-0.021 (0.036)	1, 63.5	0.34	0.564
	Ordinal Date	-0.000 (0.008)	1, 59.6	0.001	0.982
Change in Telomeres (RTM)					
	Treatment	-0.220 (0.083)	1, 17.0	7.04	**0.017**
	Age	0.054 (0.074)	1, 50.1	0.53	0.469
	Sex	-0.089 (0.067)	1, 50.8	1.77	0.189
	Clutch Size	-0.066 (0.039)	1, 11.7	2.91	0.114
	Ordinal Date	0.003 (0.002)	1, 14.0	1.39	0.257
Culmen					
	Treatment	0.181 (0.109)	1, 49.9	2.75	0.103
	Age	0.225 (0.020)	1, 352.1	112.1	**<0.0001**
	Treatment*Age	-0.036 (0.011)	1, 286.2	9.84	**0.002**
	Mass	0.095 (0.008)	1, 350.3	152.9	**<0.0001**
	Sex	0.033 (0.048)	1, 96.2	0.47	0.496
	Clutch Size	0.012 (0.044)	1, 20.0	0.08	0.780
	Ordinal Date	0.011 (0.002)	1, 20.5	19.7	**0.0002**

C		**2017 Data:**			
Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
Rectrices					
	Treatment	0.769 (1.473)	1, 94.0	0.27	0.603
	Age	3.297 (0.153)	1, 233.1	536.6	**<0.0001**
	Treatment*Age	-0.185 (0.147)	1, 150.7	1.58	0.211
	Mass	0.283 (0.064)	1, 240.2	19.1	**<0.0001**
	Sex	0.331 (0.497)	1, 73.8	0.44	0.508
	Clutch Size	-0.395 (0.477)	1, 18.1	0.69	0.418
	Ordinal Date	0.041 (0.027)	1, 18.5	2.41	0.137
Wing Chord					
	Treatment	0.156 (1.083)	1, 76.4	0.02	0.886
	Age	2.929 (0.219)	1, 346.9	188.3	**<0.0001**
	Treatment*Age	-0.148 (0.131)	1, 141.4	1.27	0.259
	Mass	0.640 (0.081)	1, 342.0	62.2	**<0.0001**
	Sex	-0.291 (0.446)	1, 332.5	0.43	0.514
	Clutch Size	0.290 (0.385)	1, 18.9	0.57	0.461
	Ordinal Date	0.036 (0.022)	1, 19.8	2.77	0.111
Pin Feathers					
	Treatment	0.663 (1.615)	1, 88.9	0.17	0.682
	Age	2.219 (0.146)	1, 194.9	271.1	**<0.0001**
	Treatment*Age	-0.184 (0.154)	1, 119.3	1.44	0.233
	Mass	-0.051 (0.065)	1, 204.1	0.61	0.437
	Sex	-0.018 (0.485)	1, 62.5	0.001	0.969
	Clutch Size	-0.249 (0.499)	1, 17.1	0.25	0.624
	Ordinal Date	0.039 (0.028)	1, 17.4	2.01	0.173
Change in Wing Web (Swelling)					
	Treatment	0.057 (0.059)	1, 15.0	0.94	0.346
	Age	0.030 (0.047)	1, 55.7	0.41	0.523
	Sex	0.041 (0.046)	1, 63.7	0.79	0.377
	Clutch Size	-0.017 (0.031)	1, 15.5	0.28	0.603
	Ordinal Date	0.002 (0.002)	1, 14.3	1.11	0.309

APPENDIX B: GENERAL LINEAR MIXED EFFECTS MODEL COMPONENTS AND A SUMMARY OF THE RESULTS ANALYZED USING RSTUDIO

Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
Ln Corticosterone Levels					
	Treatment	0.547 (0.203)	1, 25.8	7.26	**0.012**
	Time Point	2.265 (0.193)	22.7	138.1	**>0.0001**
Liver Telomerase Activity					
	Treatment	18453 (30027)	1, 15	0.38	0.548
Bone Marrow Telomerase Activity					
	Treatment	-226 (7326)	1, 16	0.09	0.765
Pectoralis Muscle Telomerase Activity					
	Treatment	177085 (91105)	1, 200	3.78	0.053
Liver Telomere Length					
	Treatment	-0.128 (0.139)	1, 7.29	0.86	0.384
Pectoralis Muscle Telomere Length					
	Treatment	0.014 (0.061)	1, 12	0.06	0.817
10 day old Telomere Length					
	Treatment	0.293 (0.157)	1, 6.77	3.47	0.106
10 day old Telomere Length					
	Treatment	-0.089 (0.164)	1, 6.62	0.29	0.602
	Bone Marrow Telomerase Activity	2.37E-6 (5.08E-6)	1, 4.95	21.5	**0.006**
	Treatment*Bone Marrow Telomerase Activity	1.94E-5 (8.49E-6)	1, 6.99	5.22	0.056
Change in Telomere Length					
	Treatment	0.207 (0.105)	1, 5.36	3.88	0.102
	Bone Marrow Telomerase Activity	1.19E-5 (3.88E-6)	1, 5.78	9.51	**0.023**
Liver Telomere Length					
	Treatment	0.083 (0.067)	1, 2.23	1.53	0.331
	Liver Telomerase Activity	-2.88E-6 (6.41E-7)	1, 2.41	20.2	**0.032**
Pectoralis Muscle Telomere Length					
	Treatment	0.064 (0.066)	1, 10	0.92	0.360
	Pectoralis Telomerase Activity	-2.85E-7 (1.59E-7)	1, 10	3.22	0.103
10 day old Mass					
	Treatment	-2.44 (0.89)	1, 7.32	7.44	**0.028**
10 day old Wing Chord					
	Treatment	-0.868 (1.68)	1, 7.42	0.27	0.620
10 day old Culmen					
	Treatment	-0.099 (0.17)	1, 7.55	0.34	0.580

Dependent Variable	*Independent Variable*	*Estimate (SE)*	*ddl*	*F*	*P*
10 day old Tarsus					
	Treatment	-0.395 (0.23)	1, 7.19	2.85	0.134
10 day old Rectrices					
	Treatment	0.275 (1.99)	1, 7.63	0.02	0.894
10 day old Pin Feather					
	Treatment	0.140 (0.91)	1, 7.04	0.02	0.882
Liver TERT Gene Expression					
	Treatment	-2.528 (2.31)	1, 7.22	1.19	0.309
Pectoralis Muscle TERT Gene Expression					
	Treatment	0.938 (0.78)	1, 7.68	1.44	0.265