A putative telomerase activator has tissue-specific effects on telomere length in a developing songbird

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ABSTRACT

There is good evidence that telomeres predict variation in health and longevity, yet it is unclear whether these patterns are causally derived from telomeres *per se*, in part because relatively little research directly manipulates telomere length during early life, when telomere shortening is most dynamic. Here, we test how the telomerase activator TA-65 (i.e., cycloastrogenol) affects telomere length in five tissues during the peak of growth in the wild tree swallow (*Tachycineta bicolor*). Following 8 days of oral TA-65 administration, chicks experienced telomere lengthening in the blood and accelerated feather growth, but no changes to mass over time. TA-65 did not affect telomere length in the brain or spleen and led to shorter telomeres in the liver and adrenals. This whole-organism experimental manipulation of telomere dynamics therefore reveals limitations to telomere protection and biological senescence. In doing so, this work advances our understanding of early-life telomere dynamics and their potential role in generating future variation in health and lifespan.

1. Introduction

Environmental perturbations during early development can set the trajectory for later deterioration of somatic function over time (i.e. senescence), and therefore may shape lifespan at a very early age (Bazopoulou et al. 2019, Boonekamp et al. 2014, Taylor 2010). In the last decade, telomere dynamics have emerged as one key mechanism that may link these early life conditions with future fitness outcomes (Price et al. 2013). Telomeres are conserved nucleotide sequences that cap the ends of chromosomes and protect genomic integrity (Blackburn 2001, de Lange 2004). In recent years, evolutionary, behavioral, and biomedical research has revealed telomeres as powerful predictors of survival and longevity across vertebrates, especially among birds (Wilbourn et al. 2018, Whittemore et al. 2019, Hares et al. 2018). Telomeres typically shorten with age during cellular replication (Levy et al. 1992, Denham et al. 2019), and this process can be accelerated during periods of growth (Reichert et al. 2015, Vedder et al. 2018) or stress exposure (Chatelain et al. 2020, von Zglinicki 2002, but see Boonekamp et al., 2017). Short telomeres are consequently believed to prompt senescence and loss of tissue function (Armanios and Blackburn 2012) but can be repaired by telomerase, the enzyme that re-builds telomeric repeats (Blackburn et al. 1989). Although high telomerase expression is limited to minimize inappropriate cell immortality (Granger et al. 2002, e.g., cancer risk, Greider 1998), telomerase may also be a vital buffer against ageing and other deleterious fitness consequences early in life when telomere loss is greatest (Salomons et al. 2009, Pauliny et al. 2012, Zeichner et al. 1999). These ideas have led to the hypothesis that telomeres and their regulatory mechanisms truly drive, rather than track, fitness outcomes. While avian research has been key to uncovering correlations between telomeres and fitness traits, this *causal* hypothesis has not been well tested because most work alters telomere length indirectly using generalized manipulations of growth or stress (Chatelain et al. 2020, Reichert et al. 2015), rather than more direct manipulations of telomere length itself.

Cycloastrogenol is a small molecule purified from the root of *Astragalus membranaceous* (Family: Fabaceae) that has emerged as a robust stimulant of telomerase activity, and it is the active ingredient in nutritional supplements like TA-65 (de Jesus et al. 2011, Reichert et al. 2014). This extract has been used in traditional Chinese medicine for millennia and in the last 10 years, has been tested in a number of human cell lines and rodent models (reveiwed in Yu et al. 2018). Telomerase activation in aged mice increases telomerase (*TERT* subunit) transcription in the liver (de Jesus et al. 2012) and repairs telomere length in mice and birds (Reichert et al. 2014), often predominantly in the shortest telomeres (de Jesus et al. 2011). These studies also show that experimental telomerase activation and subsequent telomere elongation occur alongside improvements in tissue function (e.g., feather regeneration: Reichert et al. 2014), as well as slowed ageing and longer lifespan (de Jesus et al. 2011, de Jesus et al. 2012). These findings suggest that telomerase plays a vital role in postponing senescent phenotypes in older individuals. However, comparatively less is known about how telomerase activation affects telomere dynamics early in development, despite observations that early life conditions set the trajectory for future telomere length (Boonekamp et al. 2014, Nettle et al. 2013).

Telomerase is one of several important telomere regulatory mechanisms that may causally link telomere length to later effects on organismal function, senescence, and survival. Regulation and expression of telomerase varies across the body in many mammalian systems and cultured cell lines, typically with higher levels in more replicative tissues (Forsyth et al. 2002, Liu et al. 2010), yet whether these patterns generalize to avian species is less clear. A small number of studies in birds show that telomerase activity is high early in development but decreases into adulthood, particularly so in differentiated somatic tissues (Haussmann et al. 2007, Haussmann et al. 2004, Taylor and Delany 2000, Venkatesan and Price 1998). However, these studies also show inter-specific variation in the ontogeny of telomerase activity across different tissues, e.g. high hatchling telomerase activity in bone marrow decreases with age in the zebra finch (*Taeniopygia guttata*), but not the common tern (*Sterna hirundo*) (Haussmann et al. 2007). Regardless of these complexities, current evidence suggests that avian

telomerase is not solely limited to highly proliferative tissues or periods of intense somatic growth in the same way as mammalian systems (Forsyth et al. 2002, Haussmann et al. 2007). Critically, tissue-specific telomerase activity may serve as a gateway to variation in tissue health from a very young age that ultimately influences organismal responses to early life challenges like predation, sickness, and cognitive tasks. In addition, early growth may be linked with telomere dynamics in potentially opposing ways. For instance, rapid cellular replication hastens telomere loss during development (Levy et al. 1992, Denham et al. 2019), but high levels of telomerase early in life (Haussmann et al. 2007) may promote growth (Smith et al. 2003, de Jesus et al. 2011, de Jesus et al. 2012). Previous work shows that telomerase plays a role in energetics (e.g., glucose uptake, IGF-1 pathway) and increases hair and feather growth (de Jesus et al. 2011, de Jesus et al. 2012, Reichert et al. 2014), potentially modulating important developmental milestones. Experiments that target early life telomerase activity are therefore needed to begin disentangling how telomerase regulation, telomere length itself, and early life conditions mediate variation in health and longevity.

To improve our understanding on the causal role that telomeres play in shaping later fitness outcomes, we need experimental manipulations that can alter telomere length without relying on modification to somatic growth or early life stress. To this end, we experimentally tested how the putative telomerase activator TA-65 affects growth and telomere dynamics in developing songbird chicks. TA-65 has been used successfully in many studies of adult animals or tissues (summarized above); however, to our knowledge, this supplement has not been used to manipulate telomeres in developing young, despite the vital role this life stage plays in setting future health outcomes. Our experiment used free-living tree swallows (*Tachycineta bicolor*) during the peak of early post-natal growth, a time during which chicks can grow by 4-fold in a matter of days. This period of rapid cellular proliferation typically occurs alongside higher rates of telomere loss as well as greater telomerase expression (Forsyth et al. 2002, Salomons et al. 2009), but it is unclear how telomerase activation beyond these constitutive levels affects telomere length in juveniles and how this varies among tissues. To explore these questions and facilitate future research assessing how telomere dynamics shape larger fitness outcomes like health and longevity, we measured telomere length in a suite of metabolic, immune, endocrine, and neural tissues after chicks received 8 days of oral administration of the putative telomerase activator TA-65. We expected longer telomeres in chicks given TA-65 and furthermore, that tissues would vary in the strength of this effect.

2. Materials and methods

2.1. Study subjects and experimental design

We conducted this experiment during spring and summer of 2019 in a nest box population of free-living tree swallows located near Bloomington, Indiana, USA (39.1653°N, 86.5264°W). In total, this experiment used 131 chicks distributed among 25 nests (n=5.2±0.2 chicks per nest). We used a within-nest experimental design, with half of the chicks in each nest receiving oral dosing of TA-65 (T.A. Sciences, New York City, NY) and half receiving sterilized water (control). We were not aware of a published dose for developing songbirds (elaborated in Section 2.2.), so we tested both low and high doses of TA-65, maintaining a 2-factor within-nest design. 11 nests received low vs. control (n=26 water, 30 TA-65 chicks), and 14 nests received high vs. control (n=37 water, 38 TA-65 chicks). Among nests, we balanced TA-65 dose (low vs. high) by date and brood size.

The experiment began when chicks were 4 days post-hatching (dph; hatch day=day 1). We uniquely trimmed each chick's toenails for individual identification, weighed them (nearest 0.1g), and collected 15-30µL blood from the metatarsal vein. We then assigned treatment (water or TA-65), which was counterbalanced by mass within and between nests. For 8 consecutive days (4 to 11dph), we weighed each chick and orally administered either water, a low dose (0.1mg/g) of TA-65, or a high dose (0.2mg/g) of TA-65. We visited chicks a final time on 12dph, when we (a) measured mass and wing

length, (b) banded all chicks with one numbered USGS band, and (c) collected ≤50µL blood from the alar vein, to quantify telomere attrition in blood over the study period. We selected 12dph because chicks are approaching asymptotic adult-like mass and are experiencing accelerated feather growth, both of which are linked to future survival (Magrath 1991, Martin et al. 2018, Martin et al. 2011, McCarty 2001a) and fecundity (Haywood and Perrins 1992). While in the nest, chicks continue growing feathers until and after fledging (~21dph), at which time wings are approaching adult lengths (McCarty 2001b). In addition, 12-day old chicks do not prematurely fledge in response to research activities at their nest. To measure experimental effects on telomere length in non-blood tissues, we euthanized one TA-65 and one control chick from all high vs. control nests on 12dph (n=28 chicks total, counterbalanced by mass, in n=14 nests). To minimize impact to the breeding population, chicks from low vs. control nests were not euthanized, although we recognize this design limits our ability to test dose-dependent effects on non-blood tissues. Chicks were euthanized with an overdose of isoflurane, followed by decapitation and collection of blood, brain, spleen, liver, and adrenals. Samples were snap frozen on dry ice in the field, within 11.6±0.2min of euthanasia, and stored at -80°C.

2.2. TA-65 dosing regime

Previous studies have administered TA-65 in a wide range of doses, from an estimated 0.02–0.1 mg/g body mass daily, and only some of these studies effectively manipulated telomere length (Reichert et al. 2014, de Jesus et al. 2011, Rollings et al. 2017). These studies use vertebrate classes that vary dramatically in metabolism and bone density (i.e. mice, birds, lizards), and none of the published work uses developing animals. Therefore, it was difficult to predict an appropriate dose of TA-65 for our study. We chose two doses: low (0.1 mg TA-65/g body mass) and high (0.2mg TA-65/g body mass).

For 8 consecutive days (4 to 11dph), we administered one of three treatments to each chick: water, a low dose of TA-65, or a high dose of TA-65. Because we timed our experiment to occur during the peak of nestling growth, we adjusted all dosing based on mass data compiled from locally breeding tree swallows and previous publications (McCarty and Winkler 1999, Quinney et al. 1986, McCarty 2001a). We then estimated the volume of TA-65 that would result in a low and high dose, based on the estimated average daily mass of developing tree swallows. The volume required to maintain these daily doses ranged from 115 to 392µL, and we adjusted these volumes every other day (d4-5: 115uL, d6-7: 177uL, d8-9: 328uL, d10-11: 392uL) for all three experimental groups, including water controls. Each morning, we mixed fresh TA-65 powder (obtained from commercially available capsules) with sterile water, to prepare both low and high concentration suspensions, which we administered at volumes resulting in our intended doses. This method allowed us to scale up the amount of TA-65 given as chicks grew larger, and it ensured that all treatments were given the same volume of liquid across ages. Upon completion of the study, we used population-level daily average mass from all individual chicks in this study to confirm post-hoc that the actual average doses of TA-65 received by chicks were low: 0.095 ± 0.005 mg/g and high: 0.19 ± 0.01 mg/g.

2.3. Quantification of telomere length

We quantified telomere length via quantitative PCR using methods modified from previous work (Criscuolo et al. 2009, Cawthon 2009). To begin, we extracted DNA from blood, brain, spleen, liver, and adrenals. We used the automated Maxwell® RSC Instrument (Promega, Madison, WI) with Whole Blood DNA Kit (Promega #AS1520) to extract DNA from 15µL whole blood, which was eluted into 60µL buffer. Non-blood tissues were manually homogenized on a sterile chilled block and an aliquot of the homogenate was used for DNA extraction. We used the Tissue DNA Kit (Promega #AS1610) to extract DNA from spleen, liver, brain, and one adrenal, with 80µL TE buffer, eluted into 100µL buffer. We quantified DNA concentration using the Epoch microplate spectrophotometer (BioTek, Winooski, VT).

Telomere length was measured as the ratio (T/S) of telomere repeat copy number (T) to a single gene copy number (S), relative to a pooled reference sample present on all plates. We amplified our single copy gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using primers GAPDH-F (5'-AAC-CAG-CAA-AGT-ACG-ATG-ACA-T-3') and GAPDH-R (5'-CCA-TCA-GCA-GCA-GCC-TTC-A-3'), originally developed in zebra finches (Criscuolo et al. 2009). We amplified telomeres using primers telg (5'-ACA-CTA-AGG-TTT-GGG-TTT-GGG-TTT-GGG-TTT-GGG-TTA-GTG-T-3') and telc (5'- TGT-TAG-GTA-TCC-CTA-TCC-CTA-TCC-CTA-TCC-CTA-TCC-CTA-ACA-3'), originally developed in humans but used across vertebrate taxa (Cawthon 2009). We conducted qPCR on 384-well plates (ABI Quantstudio 5, Foster City, CA). For each sample, we ran GAPDH and telomere reactions in triplicate on the same plate. Prior to plating, we diluted DNA samples to 3.33ng/µL using ultra-pure water. Each reaction had a total volume of 10µL containing 5µL PerfeCTA SYBR Green SuperMix Low ROX (Quanta Biosciences, Gaithersburg, MD, USA), 200nM each GAPDH-F/GAPDH-R or 200nM each telc/telg, and 3µL DNA extract (10ng total). qPCR reaction conditions were: 10 min at 95°C, followed by 30 cycles of 10 s at 95°C, 1 min at 62°C, and 30 s at 72°C, followed by 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. All samples fell within the bounds of the standard curve and reaction efficiencies were 98.72 ± 2.77 (GAPDH) and 107.55 ± 8.22 (telomere).

Mean Ct values (per triplicate) were used to calculate the relative T/S ratios for each sample using the following formula: $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct^{\text{telomere}} - Ct^{\text{GAPDH}})_{\text{reference}} - (Ct^{\text{telomere}} - Ct^{\text{GAPDH}})_{\text{focal}}$. Telomere length for all samples was calculated relative to a pooled reference sample obtained from tree swallow whole blood that was present on all plates. We used this reference sample to calculate the inter-plate coefficient of variation: 3.09% and 3.13% for GAPDH and telomere Ct's, respectively. The average telomere length of this pool fell within the Ct range of samples in the study. We also re-ran a subset of samples (n = 28) and found that $2^{\Delta\Delta Ct}$ values were highly repeatable across plates (Intraclass Correlation Coefficient: 0.86; 95% confidence interval: 0.73 - 0.93). For analysis of the change in whole blood telomere length over time, both samples from a single individual (4 and 12 dph), as well as all siblings from the same nest, were included on the same plate, and plates (n=5) were balanced by treatment, hatch date, and brood size. All remaining tissues (i.e., liver, spleen, brain, and adrenals) were run on separate plates. Of the 131 chicks initially in the study, 7 were excluded from qPCR analyses: n=6 did not survive to 12dph (n=1 water, n=2 low, n=3 high), and n=1 showed poor replicates in qPCR, leaving n=124 chicks with repeated measures of blood telomere length and n=28 chicks with telomere data from additional tissues.

2.4. Statistical analyses

All statistical analyses were performed in R (version 3.5.3, (R Core Team 2019)). We performed linear mixed effects models (LMMs) using the nlme package (Pinheiro et al. 2020), and reported effective degrees of freedom using the Satterthwaite method. All models initially included treatment as a 3-factor effect, but we found no differences between high and low dose chicks in any metrics and therefore combined them for all analyses (Supplementary material Appendix 1, Fig. A1, Tables A1-3).

The effect of TA-65 administration on chick growth over time (mass measured daily) was analyzed in a repeated-measures linear mixed model with treatment, age, and their interaction as fixed effects. We also included hatch date and brood size as covariables and accounted for non-linear growth by adding an age^2 term (as in Andreasson et al. 2018). To account for increasing variation in mass with age, we used the 'varFixed' function (as in Cox et al. 2019). Chick ID nested in nestbox ID was included as a random effect to account for non-independence of data, and we used an autoregressive covariance structure to control for correlations between successive measurements in each chick (Nettle et al. 2013). To quantify effects of treatment on wing length at 12-days old, we used an LMM with fixed effects of treatment, initial chick mass, hatch date, brood size, and sampling time, and random effect of nestbox ID.

We next quantified the effect of TA-65 administration on telomere dynamics. Using a repeated measures design, we first tested for treatment effects on the change in blood telomere length from 4 to 12dph using the metric *D*, which is a widely used correction in telomere biology accounting for regression to the mean (Verhulst et al. 2013). We assessed whether *D* significantly differed between chicks treated with water and TA-65 using LMM, accounting for brood size, hatch date, and qPCR plate as additional fixed effects, and nestbox ID as a random effect. To add confidence to our findings of telomere elongation, we validated that within-individual changes in blood telomere length were robust to measurement error (similar to van Lieshout et al. 2019; full details in electronic supplementary material). In similar models, we also tested for preexisting differences in telomere length between treatments, as well as final effects of treatment on blood telomere length at 12dph. Second, we compared the effects of TA-65 among non-blood tissues, where we fit a model with relative telomere length at 12dph as the dependent variable and treatment, tissue, the treatment x tissue interaction, hatch date, and brood size as fixed effects. Chick ID nested in nestbox ID was included as a random effect. Tukey post-hoc tests were run on significant interactions using lsmeans (Lenth and Lenth 2018). Blood was not included in our analysis of tissue differences at 12dph because discrepancies in sample size (i.e., n=124 blood vs n=28 non-blood) can generate unequal variances and bias results.

3. Results

TA-65 administration had no effect on mass, either as a main effect ($F_{1,105}$=0.083, p=0.77) or in interaction with age ($F_{1,1009}$=0.28, p=0.59) or age^2 ($F_{1,1009}$=0.11, p=0.74). As expected, chick mass was significantly related to age ($F_{1,1009}$=8016.08, p<0.0001) and age^2 ($F_{1,1009}$=567.01, p<0.0001), as well as brood size and hatch date (see Table 1 for complete model details, Supplementary material Appendix 1, Fig. A2). In addition, TA-65 treatment had a significant effect on wing length ($F_{1,98}$=4.79, p=0.031), where TA-65-dosed chicks exhibited longer wings relative to controls (water: 48.6±0.9 mm; TA-65: 50.3±0.7 mm). As expected, larger broods, initially smaller chicks, chicks from later-laid nests, and nests measured earlier in the morning were associated with shorter wing lengths (see Table 1 for complete model details).

Prior to treatment, chicks administered water or TA-65 did not differ in initial telomere length (β=0.053, $F_{1,95}$=1.37, p=0.24). TA-65 significantly affected the change in relative telomere length (*D*) measured in blood (β=-0.11, $F_{1,94}$=7.59, p=0.007, Fig. 1): after accounting for nestbox ID, TA-65-dosed chicks exhibited significant telomere elongation from 4 to 12-days old relative to controls, which is robust to technical error (full details in electronic supplementary material). *D* was not related to brood size ($F_{1,22}$=1.45, p=0.24), hatch date ($F_{1,22}$=0.65, p=0.43), or qPCR plate ID ($F_{1,94}$=0.57, p=0.68). Consequently, TA-65-dosed chicks ended with significantly longer blood telomere length relative to controls (β=0.085, $F_{1,94}$=4.18, p=0.044), with no significant effects of brood size ($F_{1,22}$=1.36, p=0.26) or hatch date ($F_{1,22}$=1.50, p=0.23), and a marginal plate effect ($F_{4,94}$=2.05, p=0.093).

In the subset of individuals for which we sampled non-blood tissues, telomere length responded differently to TA-65 among tissues (Fig. 2, tissue: $F_{3,78}$=7.96, p=0.0001; treatment: $F_{1,13}$=5.38, p=0.037; treatment x tissue interaction: $F_{3,78}$=3.72, p=0.015). Post-hoc Tukey tests show that telomere length at the end of the study was significantly lower in TA-65 chicks in the liver (t=2.25, p=0.043) and adrenals (t=3.27, p=0.0061), but there were no treatment effects on telomere length in the brain (t=-0.91, p=0.38) or spleen (t=0.035, p=0.97). Brood size ($F_{1,11}$=0.0019, p=0.97) and hatch date ($F_{1,11}$=0.18, p=0.68) were not related to final telomere length in non-blood tissues. In control chicks, telomere lengths were only correlated between two tissues, blood and liver (Spearman's ρ =0.70, p=0.037); TA-65 chicks showed no significant correlations in telomere length across tissues (Fig. A3.).

4. Discussion

Telomeres have emerged as powerful predictors of health and longevity in avian evolutionary and behavioral research, yet most prior manipulations of early telomere dynamics rely on modifications to somatic growth or early life stress, rather than modification of telomere dynamics per se. To our knowledge, our experiment is the first to assess the effects of TA-65 on telomere length across multiple tissues during early postnatal development. We found that 8 days of oral administration of the telomerase activator TA-65 led to significant telomere elongation in the blood. This effect occurred without significant effects on mass gain, though experimental chicks showed significant acceleration of feather growth. TA-65 may therefore provide an exciting opportunity to manipulate telomere dynamics directly with minimal manipulation of growth; critically, this is a key step in disentangling the direct effects of telomeres themselves vs. other correlated traits on the ageing process. On the other hand, we also observed shorter telomeres in the liver and adrenals of TA-65-treated chicks, suggesting that TA-65 may not globally rescue telomere length. This systems-level view of telomere dynamics offers important insights into potential constraints and consequences of telomere regulation during an age when telomerase is highly active and telomere attrition is thought to play a deterministic role in later health and longevity.

Eight days of TA-65 treatment led to significant telomere elongation in the blood; however, other tissues exhibited either no response to TA-65 (i.e., brain, spleen) or exhibited shorter telomere length relative to controls (i.e., liver, adrenals). At this time, little is known about the ontogeny and tissue-specificity of telomerase activity among birds, especially as it compares to patterns shown in non-avian systems (reviewed in Swanberg et al. 2010; Taylor and Delany 2000). Prior mammalian work shows that experimental upregulation of telomerase activity delays ageing and rescues cognitive and reproductive function in adult mice, among other apparent benefits (Tomás-Loba et al. 2008, de Jesus et al. 2011, de Jesus et al. 2012, Jaskelioff et al. 2011). Interestingly, the subunits of telomerase themselves, i.e., the reverse transcriptase (TERT) and RNA template (TERC), also mediate ageing pathways independent of telomerase activity (Geserick and Blasco 2006, Cong and Shay 2008). Constitutive variation in these subunits across tissues may therefore influence the potential for further increase in telomerase activity, as well as the extent of physiological benefits received due to ceiling or hormetic effects. Tissues exhibiting high or increased levels of telomerase (e.g., higher levels in brain vs. liver of postnatal tree swallows (Haussmann et al. 2007)) may be more likely to develop cancers (Granger et al. 2002, Greider 1998), although the extent of hormetic effects may vary by age or progression of cellular senescence. Repair of telomeres by TA-65 in mice occurs predominantly in short telomeres (de Jesus et al. 2011), which may experience fundamentally different regulation than longer counterparts (Nordfjäll et al. 2009). Notably, we show that the tissue with the shortest telomere length in control chicks (i.e., blood) experienced TA-65-induced *elongation* whereas the tissue with the longest telomere length in control chicks (i.e., liver) ended the experiment with significantly *shorter* telomeres after TA-65 treatment. Given that telomeres often reflect tissue functionality (Armanios and Blackburn 2012), telomere loss in the liver and adrenals could impact survival-oriented processes that negate the otherwise positive effects of telomerase activation in other tissues. A full understanding of these complexities would benefit from further dose-dependent analyses in non-blood tissues, as well as longitudinal analyses to assess how telomerase activation influences suites of fitness-related traits, beyond the two morphological traits assessed here.

Effective manipulation of telomere dynamics themselves will play a critical role in furthering our understanding of the causal links between telomeres and fitness, and the tissue-specific effects we identified contribute to this long-term goal. Several non-mutually exclusive hypotheses could further explain this tissue variability. Oral administration may limit which tissues are exposed to the drug (i.e., compared to viral vectors: de Jesus et al. 2012), although previous work shows marginal effects of TA-65 on telomerase across many tissues in adults, including the brain (de Jesus et al. 2011). Alternative

splicing of telomerase (*TERT* subunit) transcripts can also vary among tissues and can produce inactive forms of telomerase (Ulaner et al. 2000, Liu et al. 2017, Jie et al. 2019), suggesting some tissues may be more amenable to pharmacological manipulation of telomerase activity. Lastly, extensive liver metabolism of TA-65 could accrue cytotoxic effects (Zhu et al. 2010) leading to shorter liver telomere lengths seen in TA-65 chicks compared to their sibling controls. Regardless of the exact mechanism, these tissue-specific effects speak to potential trade-offs in which gains in telomere length in some tissues may be offset by losses elsewhere in the body, adding credence to the view that some organismal systems may be more prone to biological ageing than others (Tuttle et al. 2020). Furthermore, these results highlight the need to quantify telomere dynamics across tissues rather than solely in blood as we continue to explore the central driving role of telomeres in health and longevity.

TA-65-treated chicks exhibited significantly longer feathers by 12-days old, consistent with previous work showing that telomerase activation enhances feather and hair growth (de Jesus et al. 2011, de Jesus et al. 2012, Reichert et al. 2014). Telomerase (i.e., TERT subunit) performs a telomere-independent role in cellular replication across taxa (Marión and Blasco 2010, Chiodi and Mondello 2012, Gomez et al. 2012): overexpression in mice promotes proliferation of epidermal stem cells (Flores et al. 2005, Sarin et al. 2005, Tomás-Loba et al. 2008) and in amphibians and lizards, telomerase is implicated in wound healing and limb regeneration (Seifert et al. 2012, Alibardi 2015b, Alibardi 2015a). Little comparable work exists for passerine species; however, telomerase is localized in embryonic chicken skin (Alibardi 2020) and is associated with cell signaling in feather development (Park et al. 2009), suggesting a similar role in birds. These advancements in feather growth could accelerate time to fledging and decrease predation risk while in the nest (Martin et al. 2018, Martin et al. 2011). On the other hand, tree swallow wings grow 4-6mm per day at this age (McCarty 2001b), and so the 1.7mm difference we observed could accrue in roughly 8 hours, meaning it may have limited biological relevance. How these changes in feather growth interact with other, potentially deleterious effects of activated telomerase is as of yet unclear. However, these relatively mild advances in feather growth, coupled with the null effects of TA65 on body mass, suggest that this drug has some promise for isolating the causal links between telomere dynamics and fitness.

In summary, we found that 8 consecutive days of TA-65 administration in developing chicks led to telomere elongation in the blood but surprisingly, also led to shorter telomeres in the liver and adrenals. This effect was independent of manipulations to body mass and controlled for age, opening new doors into our ability to understand telomeres and their repair mechanisms as potentially causative agents in health and longevity and important players in evolutionary ecology of birds. It is especially important to understand these dynamics in developing juveniles, where telomere dynamics have the potential to set individuals on trajectories of senescence over their lifetime.

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MANUSCRIPT FIGURES

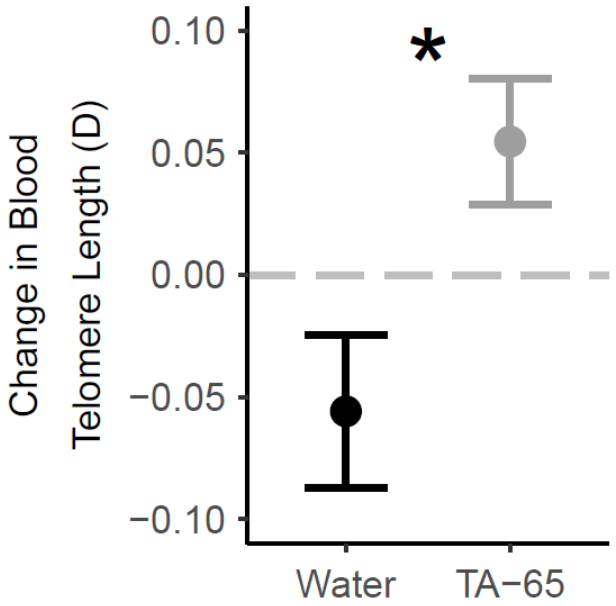


Fig. 1. The change in blood telomere length, corrected for regression to the mean (*D*), from 4 to 12 days post-hatch in chicks that were orally administered water (black, n=61) or TA-65 (n=63, gray). The dashed line represents no change in telomere length, and positive/negative values denote telomere elongation and shortening, respectively. Data are plotted as mean ± SE.

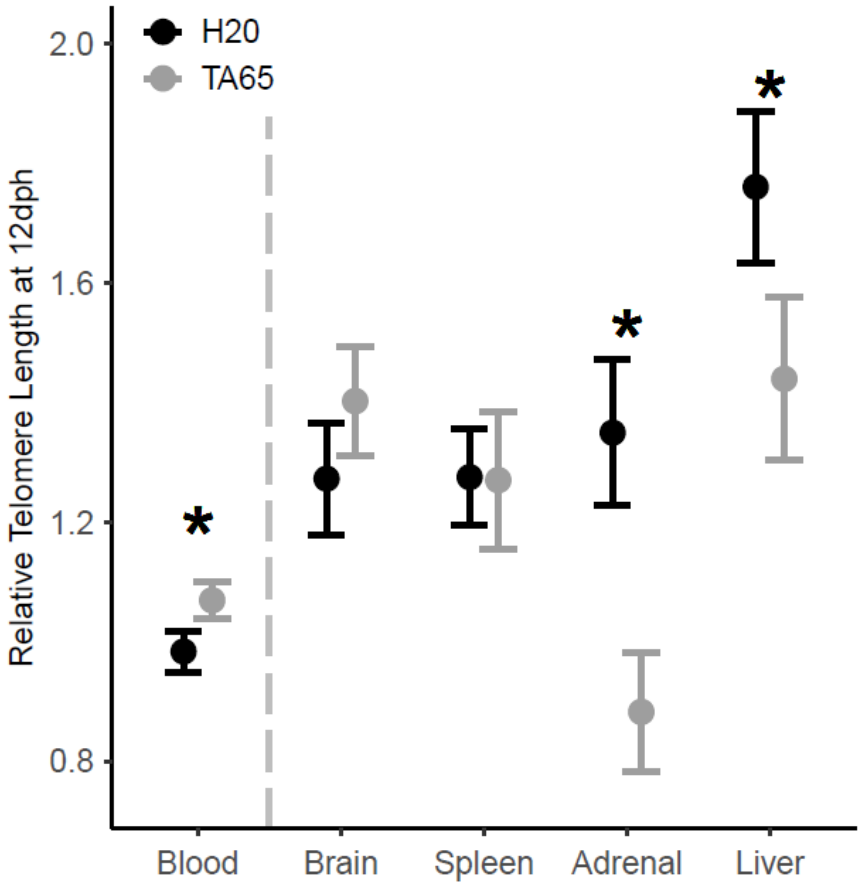


Fig. 2. Relative telomere length of 4 tissues collected from 12-day old chicks orally administered either water (n=28, black) or TA-65 (n=28, gray) for 8 consecutive days. Note that blood telomere length (n=61 water, n=63 TA-65) was statistically analyzed separately from non-blood tissues, but we replot these data here for reference. Data are plotted as mean ± SE.

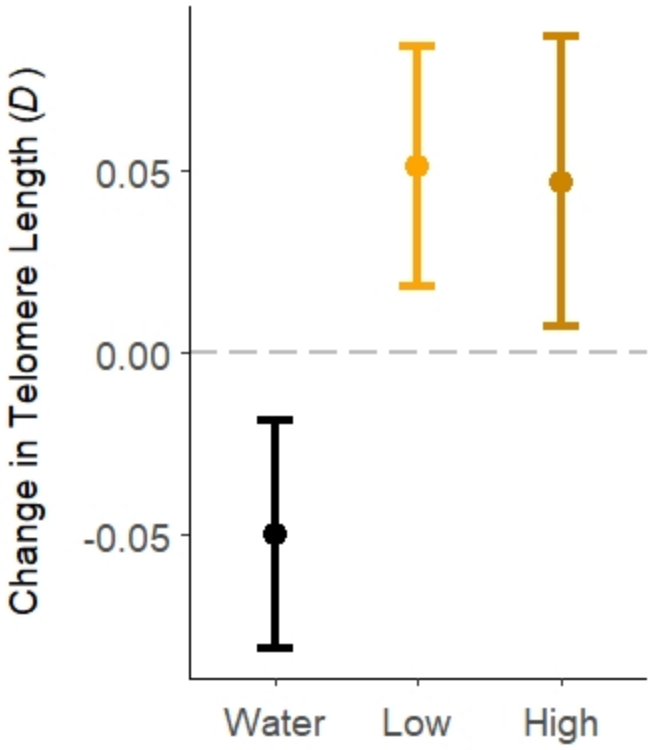
Table 1. Summary of mixed models testing for the effects of oral TA-65 on growth variables: chick mass measured over the 8 days of treatment (n=131 chicks) and wing length measured at the end of the study (n=125 chicks), across 25 nests. Effective degrees of freedom (DF) are corrected using the Satterthwaite method, and significant effects (p<0.05) are reported in bold.

Fixed Effects	Estimate ± SE	Effective DF	F-value	P-value
Response variable: chick mass measured daily from 4-12 days old				
Random effect: chick ID nested within nestbox ID				
Intercept	-7064.54 ± 1592.10	1, 1009	1239.72	**<0.0001**
Age	4.06 ± 0.14	1, 1009	8016.08	**<0.0001**
Age^2	-0.14 ± 0.01	1, 1009	567.01	**<0.0001**
Treatment	0.15 ± 0.71	1, 105	0.08	0.77
Brood Size	0.80 ± 0.28	1, 22	6.36	**0.02**
Hatch Date	0.00 ± 0.00	1, 22	19.61	**0.0002**
Treatment*Age	0.08 ± 0.19	1, 1009	0.28	0.59
Treatment*Age^2	-0.004 ± 0.01	1, 1009	0.11	0.74
Response variable: wing length at 12-days old				
Random effect: nestbox ID				
Intercept	7012.18 ± 3428.77	1, 98	7871.41	**<0.0001**
Treatment	0.20 ± 0.49	1, 98	4.79	**0.03**
Initial Mass	3.86 ± 0.28	1, 98	187.91	**<0.0001**
Brood Size	-1.02 ± 0.58	1, 21	4.88	**0.04**
Hatch Date	0.00 ± 0.00	1, 21	9.70	**0.005**
Sampling Time	31.91 ± 14.77	1, 21	4.67	**0.04**

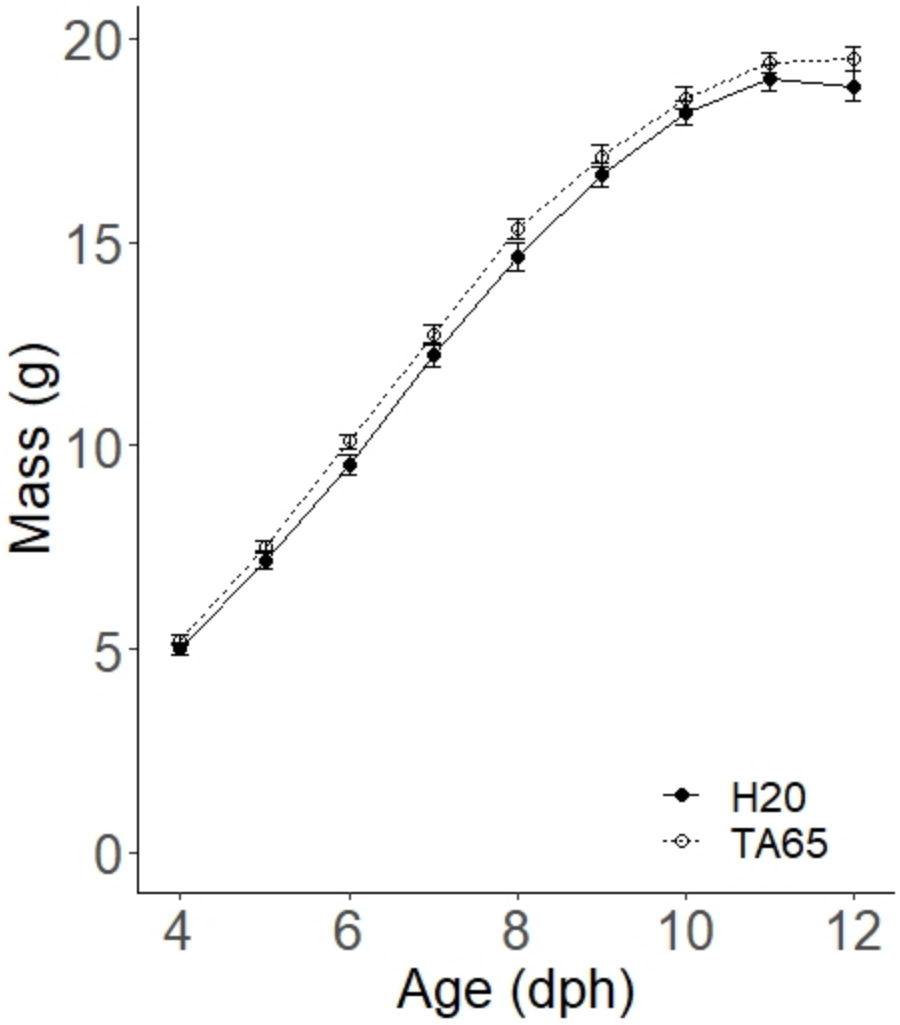
ELECTRONIC SUPPLEMENTARY MATERIAL

A putative telomerase activator has tissue-specific effects on telomere length in a developing songbird

Telomere Lengthening Validation*:* 40% of chicks in our study (n = 50/124) exhibited an increase in telomere length in blood, from 4 to 12-days old. Sporadic telomere elongation is reported in many studies but has traditionally been attributed to measurement error (Bateson and Nettle 2017, Steenstrup et al. 2013), so we performed additional analyses to demonstrate that our results are robust to technical variation. To test if the magnitude of change in blood telomere length from 4 to 12-days old was greater than measurement error in technical replicates, we a) estimated variation among repeated samples of individuals over time and compared it to b) variation in relative telomere length among triplicates on a plate. Modified from van Lieshout et al. (2019), we used MCMglmm (Hadfield 2010) with an inverse Wishart prior (*v*=1, nu=0.002; iterations=600,000, thin=300, burn-in=15,000). Using repeated estimates of telomere length at 4 and 12dph, our first model (a) predicted telomere length with individual ID and qPCR plate ID as random effects (n=248 samples; 124 individuals). We then randomly selected one set of triplicates per individual (4 or 12dph) and constructed a second model (b) with telomere length for each of the technical replicates as the response variable and individual ID as a random effect (*n*=124 individuals); this model essentially assesses intra-replicate variation. We then compared the variance explained by individual ID between these two models and assessed whether the 95% confidence intervals overlapped. When accounting for plate effects using MCMCglmm, the random effect estimate for individual ID with technical replicates was 0.069 (95% CI = 0.051, 0.088), whereas for within-individual samples the random effect estimate was 0.033 (95% CI = 0.016, 0.049). The 95% CI's from the technical replicates and within-individual samples did not overlap, suggesting that telomere elongation in blood following TA-65 administration cannot be explained by measurement error alone.



578 **Fig. A1.** Change in blood telomere length (*D*) from d4 to d12, analyzed as a 3-factor treatment
579 comparing chicks that were orally administered water (n=61), low TA-65 (n=28), or high TA-65 (n=25) for
580 8 consecutive days. Dashed line represents no change in telomere length, and positive/negative values
581 denote telomere elongation and shortening, respectively. Error bars are ± SE.



582 **Fig. A2.** Growth curve from 4 to 12dph in tree swallow nestlings given either water or TA-65 (n=131
583 chicks). Maximum growth occurs at 6.1±0.1 days. Error bars are SE.

Table A1. Output for repeated measures model predicting chick mass, analyzed as a 3-factor treatment: water, low TA-65, and high TA-65-treated chicks over the 8 days of the experiment (n=131 chicks). The model includes a random effect of chick ID nested within nest ID and controlled for increasing variance in mass with age ('FixedVar' function), as well as correlations between successive mass measurements (autocorrelation covariance structure). Effective degrees of freedom are corrected using the Satterthwaite method, and significant effects (p<0.05) are reported in bold.

Fixed Effects	Estimate ± SE	Effective DF	F-value	P-value
Intercept	-6879.53	1, 1007	1221.25	**<0.0001**
Age	4.06	1, 1007	8131.26	**<0.0001**
Age^2	-0.14	1, 1007	569.38	**<0.0001**
Treatment	(high): -0.45 ± 0.84 (low): 0.89 ± 0.93	2, 104	0.97	0.38
Brood Size	0.80	1, 22	5.81	**0.03**
Hatch Date	0.00	1, 22	17.97	**0.0003**
Treatment*Age	(high): 0.18 ± 0.22 (low): -0.04 ± 0.24	1, 980	2.77	0.06
Treatment*Age^2	(high): -0.1 ± 0.01 (low): 0.01 ± 0.02	1, 980	0.95	0.39

Table A2. Output for experimental effects of TA-65 on wing length at the end of the study in 12dph chicks, split into 3 treatments: water, low TA-65, and high TA-65-treated chicks (n=125 chicks). The model includes a random effect of nest ID. Effective degrees of freedom are corrected using the Satterthwaite method, and significant effects (p<0.05) are reported in bold.

Fixed Effects	Estimate ± SE	Effective DF	F-value	P-value
Intercept	7029.40 ± 3497.80	1, 97	7831.05	**<0.0001**
Treatment	(high): 0.21 ± 0.64 (low): 0.19 ± 0.71	2, 97	2.38	0.10
Initial Mass	3.86 ± 0.29	1, 97	186.30	**<0.0001**
Brood Size	-1.02 ± 0.59	1, 21	4.86	**0.04**
Hatch Date	0.00 ± 0.00	1, 21	9.81	**0.005**
Sampling Time	31.83 ± 15.05	1, 21	4.48	**0.05**

Table A3. Output for experimental effects of TA-65 on the change in telomere length (*D*) from 4 to 12dph, split into 3 treatments: water, low TA-65, and high TA-65-treated chicks (n=124 chicks). The model included a random effect of nest ID. Effective degrees of freedom are corrected using the Satterthwaite method, and significant effects ($p<0.05$) are reported in bold.

Fixed Effects	**Estimate ± SE**	**Effective DF**	**F-value**	**P-value**
Intercept	-97.87 ± 134.47	1, 93	0.0009	0.98
Treatment	(high): 0.10 ± 0.05 (low): 0.12 ± 0.05	2, 93	3.81	**0.03**
Plate	(P2): 0.07 ± 0.07 (P3): 0.06 ± 0.06 (P4): 0.04 ± 0.06 (P5): 0.09 ± 0.07	4, 93	0.58	0.68
Brood Size	-0.03 ± 0.02	1, 22	1.65	0.21
Hatch Date	0.00 ± 0.00	1, 22	0.41	0.53
Tukey Post-Hoc				
Contrasts	**Estimate**	**DF**	**t-ratio**	**P-value**
Water-Low	0.12 ± 0.05	93	-2.279	0.06
Water-High	0.10 ± 0.05	93	-2.056	0.10
Low-High	-0.02 ± 0.06	93	-0.331	0.94