DR. ELIZABETH SHELDON (Orcid ID : 0000-0001-5002-3006)

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Associations between DNA methylation and telomere length during early life: insight from wild zebra finches (*Taeniopygia guttata)*

Telomeres and epigenetics in a wild bird

Elizabeth L. Sheldon[1*], Riccardo Ton[1], Winnie Boner[2], Pat Monaghan[2], Shirley Raveh[2], Aaron W. Schrey[3], Simon C. Griffith[1]

[1]Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia.
[2]Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK.
[3]Department of Biology, Georgia Southern University, Armstrong Campus, Savannah, Georgia, USA

***Correspondence:** Elizabeth L. Sheldon, Department of Biological Sciences, Macquarie University, Sydney, Australia.
Email : elizabeth-louise.sheldon@students.mq.edu.au

Abstract

Telomere length and DNA methylation (DNAm) are two promising biomarkers of biological age. Environmental factors and life history traits are known to affect variation in both these biomarkers, especially during early life, yet surprisingly little is known about their reciprocal association, especially in natural populations. Here, we explore how variation in DNAm, growth rate, and early-life conditions are associated with telomere length changes during development. We tested these associations by collecting data from wild, nestling zebra finches in the Australian desert. We found that increases in the level of DNAm were negatively correlated with telomere length changes across early life. We also confirm previously documented effects of post hatch growth rate and clutch size on telomere length in a natural ecological context for a species that has been extensively studied in the laboratory. However, we did not detect any effect of ambient temperature during developmental on telomere length dynamics. We also found that the absolute telomere length of wild zebra finches, measured using the in-gel TRF method, was similar to that of captive birds. Our findings highlight exciting new opportunities to link and disentangle potential relationships between DNA based biomarkers of aging, and of physiological reactions to environmental change.

Keywords: Temperature effects, Life history, Development, Biomarkers, Aves, Aging

INTRODUCTION

Telomeres are highly conserved, non-coding DNA sequences that form protective caps at the end of eukaryotic chromosomes (Blackburn,1991, Blackburn & Epel 2012). In the absence of telomerase (a reverse transcriptase that adds telomeric repeats de novo after each cell division), telomeres shorten with each round of cell division (Harley, Futcher, & Greider, 1990). When a critical length is lost, telomeres become dysfunctional and cells enter a state of replicative senescence (Hornsby, 2003; Verdun & Karlseder, 2007, see also Victorelli & Passos, (2017) for length independent damage to telomeres triggering cell senescence). The accumulation of senescent cells is known to contribute to age-related declines in tissue and organ function (Wong et al., 2003). Accordingly, within species, relatively short telomeres and accelerated rates of telomere shortening have been associated with fitness costs, predominantly via reduced lifespan at the individual level (Monaghan, 2010; Eastwood et al., 2019; Boonekamp, Mulder, Salomons, Dijkstra, & Verhulst, 2014, Wilbourn et al., 2018). Given the relationship with lifespan, telomeres have been widely used as biomarkers of biological age (Jylhava, Pedersen, & Hagg, 2017), which has led to a recent focus in understanding how inter-individual variation in telomere length arises. Early life telomere length is partly determined by genetic factors (Olsson et al., 2011; Dugdale & Richardson 2018), however, accumulating evidence suggests that environmental cues also impact telomere length dynamics across the life course (Kotrschal, Ilmonen, Penn, 2007; Vedder, Verhulst, Zuidersma, & Bouwhuis, 2018; Dupoue et al., 2017; Boonekamp, et al., 2014). Information on the relationship between environmental and telomere length variation is steadily increasing, yet surprisingly little is known about whether other environmentally induced DNA modifications mirror telomere dynamics. Insight on how telomere length can vary in concert with other genetic traits can expand our understanding of DNA based biological markers of aging, and of physiological reactions to environmental change.

DNA methylation (DNAm) is an epigenetic mechanism that appears to be an important component of telomere length regulation (Blasco, 2007) and is also considered a promising biological clock (Horvath & Raj 2018). Yet, the relationship between DNAm and telomere length has not yet been explored in an ecological context in any species, although a few studies have indicated some conceptual connections (Horvath et al 2018; Blasco, 2007). DNAm usually refers to the addition of a methyl group to a cytosine base at a CG dinucleotide (a 'CpG' loci) on the DNA sequence, but can occur at other loci (Anglers et al., 2010). When DNAm occurs at a CpG loci close to a gene regulatory region, it can modulate phenotypic variation through its effects on

gene expression. Evidence suggests that DNAm could be involved in two, key telomere regulatory processes; those involving telomerase (Buxton et al., 2014), and 'alternative mechanisms' relying on homologous recombination between telomeric sequences such as alternative lengthening of telomeres (Gonzalo et al., 2006). While telomeres themselves do not contain CpG loci, subtelomeres do contain CpG sites with the potential for methylation, and decreases in global and subtelomeric DNAm are known to be concomitant with increased homologous recombination between telomeric sequences and dramatically elongated telomeres in mouse cells (Gonzalo et al., 2006). Additionally, a negative relationship between telomere length and genome-wide (Lee et al., 2019) and gene-specific (Lee et al., 2019; Buxton et al., 2014) DNAm has been described in humans (however, this relationship may be complicated as a positive relationship has been detected in a different correlative study (Dong et al., 2018)). In addition to the potential for epigenetic mechanisms to affect telomere length, evidence also suggests that telomere length may affect epigenetic mechanisms. For example, short telomeres have been shown to contain specific epigenetic marks (histone modifications) that facilitate their preferential elongation in mice (*Mus musculus*) (Hemann, Strong, Hao, & Greider, 2001; de Lange, 2005).

There is also plenty of scope for indirect relationships between DNAm and telomere length to occur given that they are both independently identified as molecular measures of age and ageing (Lu et al., 2019; Banszerus, Vetter, Salewsky, König & Demuth, 2019), and are associated with a range of the same biological and ecological factors (Feil & Fraga, 2012; Monaghan, 2014), particularly during early life (Watson, Bolton & Monaghan, 2019; Boonekamp et al., 2014). For example, studies have detected associations between DNAm or telomere length and clutch/brood size (Noguera & Velando, 2020; Jimeno, Hau, Gomez-Diaz, & Verhulst, 2019; Sheldon, Schrey, Ragsdale & Griffith 2018; Nettle et al., 2016; Reichert et al., 2014; Costanzo et al., 2016; Boonekamp et al., 2014); ambient temperature (Stier, Metcalfe & Monaghan, 2020; Sheldon, Schrey, Hurley, & Griffith, 2020; Yan et al., 2015); and body size/growth rate (Young et al., 2017; Vedder et al., 2018). It is therefore important to account for these influences when testing for potential associations between DNAm and telomere length.

The present study examined the relationship between DNAm and telomere length dynamics in a species in the wild in which individuals are exposed to natural variation in early life conditions. Specifically, we collected longitudinal measures of telomere length (using qPCR) and longitudinal measures of the level of DNA methylation across a consistent subset of loci (using methylation sensitive- amplification fragment length polymorphisms (MS-AFLP)) across the early

life of wild zebra finches (*Taeniopygia guttata*). Although MS-AFLP analyses do not provide inference on the functional, gene regulatory consequences of DNAm differences, they do provide a useful tool to compare the relationship between telomere length dynamics and changes in the percentage of a consistent subset of CpG loci that are methylated among individuals (Schrey et al., 2013). To date, associations between telomere length and DNAm dynamics have not been assessed in wild animals, and results from the few correlative, clinical studies that do exist have been mixed (Dong et al., 2018; Lee et al.,2019). However, here we predict that a negative association between DNAm/changes in the level of DNAm and telomere length/telomere length change will exist, given that laboratory experiments have implicated negative, regulatory effects of DNAm on telomere length (Buxton et al., 2014; Gonzalo et al., 2006). Across development, we also predict that levels of DNAm will increase as the methylome establishes (Watson et al., 2019; Sheldon et al., 2018), and telomeres will decrease in length with each round of somatic cell division (Watson; Bolton & Monaghan, 2015). Our study aims to provide an initial exploration into the relationship between DNAm and telomere length dynamics during development in wild animals, as well as potential associations with ambient temperature and life history effects. In doing so, we aim to establish a base for future ecological research to link and/or disentangle the relationship between early life conditions, DNA based biomarkers of age, and individual fitness parameters.

In addition to considering the links between telomere length and DNAm, our study is also of value in providing the first investigation of telomere length dynamics in *wild* zebra finches (*Taenopygia guttata)*. Characterisations of telomere dynamics in the wild have become increasingly common for a wide range of bird species. In contrast, laboratory studies on birds are largely performed using the zebra finch, in which paradoxically, telomere dynamics have yet to be explored in the wild. Studies of ecologically relevant populations of wild zebra finches are thus of value in helping to contextualize and interpret the controlled laboratory tests that have made such a significant contribution to our understanding of telomere biology in birds.

MATERIALS AND METHODS

Field work and blood sampling

Fieldwork was conducted at Fowlers Gap Arid Research Station, in far-western New South Wales (31°05’S, ‘141°42’E) during the main part of the Austral breeding season (August–December) in 2016. Data were collected from zebra finch nestlings in nest boxes in their natural habitat at

'Fowlers Gap' (details regarding field site characteristics can be found in Griffith, Pryke, & Mariette, 2008). Given the mobility of the species in the wild, we were working with a population in which most adults were not banded, and we were unable to ascertain parents' age or reproductive history. Nest boxes were monitored periodically (every 2 days) during the nest-building stage, and after the first egg was laid nest boxes were monitored each morning until the entire clutch was laid, enabling us to ascertain clutch size and projected hatch date. In the wild, we have previously shown that parent zebra finches only initiate incubation on the day that the last egg is laid (Gilby, Mainwaring, & Griffith, 2013). Consequently, we used the day of the last laid egg to represent the first day of embryonic development and the usual incubation period of ~12 days after the onset of incubation, to predict clutch hatch date. Two days prior to a clutch's estimated hatch date, we monitored the nest three times per day between 06.00 and 17.00; our first nest check was at ~06.00, our second nest check was at ~11.30 and our third nest check was at ~17.00, which allowed us to identify the hatch date of each nestling. The hatch date was used to calculate the post-hatch age of each nestling (in days). Synchronous hatching meant that we could not tie nestlings to specific eggs, we were unable to account for the order in which eggs/nestlings were produced, something that has been shown to impact telomere dynamics in the zebra finch (Noguera, Metcalfe, Reichert & Monaghan, 2016).

To measure the ambient developmental temperature of each nest, we obtained hourly atmospheric temperature data in the shade (i.e., air temperature) from the Australian Bureau of Meteorology's automated weather station at Fowlers Gap, located within 16.9km of the study site. All nests were in close proximity, within a circular area of homogenous habitat at similar elevation spanning a maximum of 2.6km in diameter. Thus, it is unlikely that any differences in temperature among nest sites could influence our results. Furthermore, the weather station is at a similar elevation to our study site (study site: 155.9m; weather station: 183m) and a strong relationship between ambient temperature at the weather station and temperature in the nest boxes has been demonstrated in an earlier study (Griffith et al., 2016).

We calculated the mean temperature during each day of zebra finch development using hourly data from 7am to 7pm. These data represent the period of the day when the adults are periodically away from the nest foraging together (Mariette & Griffith 2015), and the nestlings (or embryos) are therefore subject to the greatest exposure to ambient (rather than brooding/ incubation) temperatures; the duration of nestling exposure to ambient temperature increases as the nestlings grow, and parents reduce brooding. The daily temperature values were then averaged

from day 3 to day 11 of post-hatch development (prior to day 3, the ectothermic nestlings are typically brooded or incubated for long periods during the day, thus are not exposed to ambient temperatures, day 3 was also the age at which the earliest blood sample was taken, see below). During our sampling period (Aug-Nov), average temperatures across post-hatch development ranged from 14.6- 27.6^{o}C (average 20.3^{o}C, SEM: 0.295). At day 3 a small patch of down feathers was trimmed from a different area on each nestling allowing us to identify individual nestlings throughout development. Trimming the small patch of hair (approximately 0.5cm^2) from each nestling was unlikely to impose thermoregulatory costs.

At day 3 and day 11, we extracted blood (<20µl) from the metatarsal vein of younger (day 3) nestlings, and the brachial vein of older (day 11) nestlings with a hypodermic needle and capillary tube. Blood was preserved in 0.5ml of 95% ethanol and stored at room temperature in an Eppendorf tube that was labelled with a unique ID. Growth rate (a measure of cell proliferation) can affect telomere dynamics (Boonekamp et al., 2020; Ringsby et al., 2015). To account for variation in growth rate between individual nestlings and broods we measured tarsus length (a reliable metric of body size) at day 3 and day 11 using digital calipers. The associated effects of clutch and brood size (i.e. parental care and resource acquisition) can also affect telomere length (Costanzo et al., 2016; Boonekamp et al., 2014) and DNAm changes (Sheldon et al., 2018), thus, we included clutch and brood size in our model. We collected data on the clutch size of each nest daily from the day of first lay to the onset of incubation, and on the two days prior to the estimated day of hatch (clutch size ranged from 2 to 11 eggs). We collected data on the brood size of each nest on the day the entire clutch hatched, day 3, and day 11 of post-hatch development (brood size ranged from 2 to 8 nestlings). Due to egg/nestling mortality, clutch/brood size often varied across development, thus we averaged the clutch/brood size of each nest across the two time-points.

Sample size

Two blood samples were available for analysis from 91 zebra finches from 62 nests - a total of 182 samples. 73% of these samples yielded enough DNA for assaying telomere length successfully using qPCR – a total of 133 samples from 83 individuals from 57 nests, 50 of these individuals from 38 nests were measured successfully for telomere length at both day 3 *and* day 11. Of the 83 individuals analysed successfully for telomere length, 78 individuals from 60 nests, a total of 106 samples, were analysed successfully for MS-AFLP. 28 of these individuals from 23 nests were measured successfully for MS-AFLP at both day 3 *and* day 11. Generally, only a very

small blood sample could be collected from the <3g, day 3, nestling zebra finches. Sample failures during qPCR analysis were generally due to an insufficient DNA yield from a small, initial blood sample. Sample failures during MS-AFLP analysis were generally due to the sensitivity of the MS-AFLP protocol to DNA concentration during the restriction digest step – where inconsistencies in initial concentrations of DNA extract meant that the enzymes did not cut optimally in all samples. This meant that a subset of the subsequent PCRs failed. Additionally, two reactions (MspI and HpaII) must be compared for every individual and if one reaction fails, methylation cannot be scored for that sample. Consequently, it is unlikely that underlying, biological differences exist between samples that were scored successfully and those that failed. Additionally, it is unlikely that our storage method of whole blood (in ethanol) affected our molecular analyses, given that this storage technique has been used successfully in other studies to store whole blood over longer time periods (Barret et al., 2013; Nussey et al., 2014). Due to logistical constraints during field work, tarsus length and/or clutch size was not recorded for six nestlings from five nests, leading to the final sample sizes reported in Tables 1 and 2.

DNA methylation measurements

The MS-AFLP protocol used modifies the standard AFLP protocol by substituting the MseI enzyme with the methylation-sensitive isoschizomeric enzymes MspI and HpaII (New England Biolabs). See Salmon et al., (2008) for further details on the MS-AFLP method. Together, four types of variation can be scored: Type 1 is when both enzymes cut at the restriction site and indicates no methylation, Type 2 is when MspI does cut and HpaII does not cut, indicating the restriction site has a methylated internal cytosine C; Type 3 is when MspI does not cut and HpaII does cut indicating the restriction site has hemi-methylation; and Type 4 is when neither enzyme cuts indicating either both cytosines are methylated or the restriction site has mutated (Richards, Schrey & Pigliucci, 2012) (conservatively, we treated Type 4 as missing data because the underlying methylation state cannot be determined; for example see Richards et al., 2012).

We performed MS-AFLP following the protocol used by Richards et al., (2012). For the MS-AFLP analysis, DNA was extracted using the Gentra Puregene tissue kit (Qiagen, Valencia, CA, USA) and was stored in 40 µl of TE buffer. For both day 3 and day 11 samples, we digested approximately 250ng of genomic DNA at 37º C for 3 h in paired reactions: one with EcoRI and MspI, the other with EcoRI and HpaII. We immediately followed the restriction digest with adaptor ligation with EcoRI and MspI/HpaII adaptors at 16-20 h at 16º C (Supplementary Material

Table 1, all primer and adapter sequences). After adaptor ligation, we conducted pre-selective PCR with EcoRI+1, MspI/HpaII+0 pre-selective primers (Supplementary Material Table 1) at the following PCR conditions: 75º C for 2 min; 20 cycles of 94º C for 30 s, 56º C for 30s, 75º C for 2 min, final extension at 60º C for 30 min and 4º C hold. Following pre-selective PCR, we conducted selective PCR by multiplexing 6-FAM fluorescently labelled EcoRI+AGC primers with HEX fluorescently labelled EcoRI+ACG primers and unlabeled primers HpaII/MspI+TCAT (Supplemental Table 1) at the following PCR conditions 94º C for 2 min, 8 cycles of 94º C 30 s, 65º C 30 s 72º C 2 min (dropping the annealing temperature 1º each cycle), 31 cycles of 94º C 30 s, 56º C 30 s 72º C 2 min, final extension of 60º C 5 min and a 4º C hold. We sent the selective PCR products to Macrogen Facilities (South Korea) for fragment analysis on an ABI 3130XL.

We used PEAKSCANNER v 1.0 (Applied Biosystems) to analyse resultant gel files and define fragment sizes, and RAWGENO (Arrigo et al., 2012) to define bands. We pooled data into two categories: methylated (Type 2 and Type 3) or not methylated (Type 1). Throughout, we refer to a MS-AFLP locus to indicate a particular sized band resolved in the selective PCR. To ensure scores were consistent, we validated our MS-AFLP results by duplicating the entire protocol for 30 random individuals. We identified bands that consistently occurred, and we eliminated bands that were inconsistently amplified or occurred at highly variable intensities. We conducted all analyses using a binary haplotype-binding pattern (methylated 1, not methylated 0) for 92 verified, consistent CpG loci between 50 and 500 base pairs for each nestling. We calculated percentage of DNAm as the proportion of the 92 loci that were methylated for each sample, we refer to this throughout as DNAm.

Telomere measurements

Genomic DNA (gDNA) was extracted using the NucleoSpin blood kit (Macherey-Nagel) with some minor modifications. 2 µl of whole blood was removed from the sample tube and allowed to air-dry, to evaporate the ethanol, and subsequently added to 198µl of PBS. From this stage, the manufacturer's protocol was followed, with the gDNA eluted into 35µl of BE buffer. The quantity and purity of the gDNA was measured on a Nanodrop 8000 and all samples were within the accepted parameters; $A_{260/280} \geq 1.7$, $A_{260/230} \geq 1.8$ (Thermo Fisher). The gDNA was stored at -20ºC until telomere length analysis was performed.

Telomere length was assayed using the qPCR method as described previously (Criscuolo 2009). Briefly, the telomere length of each sample was measured by determining the ratio (T:S) of

telomere repeat copy number (T) to a single copy or non-variant control gene (S), relative to a five pooled DNA reference sample from wild zebra finches at day 11 that was run on all plates. For this study, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control gene. A standard curve (6 serial dilutions of zebra finch gDNA from 40 to 1.25ng/well) was also included on each plate and all samples (ran in triplicate) fell within standard curve boundaries. Mean reaction efficiencies were within the acceptable range for telomere and control gene (mean ± SE, TEL: 97.43 ± 4.54%; GAPDH: 87.73 ± 3.75%). The average inter-plate coefficient of variation of the Ct values was 1.47 for the telomere assay and 2.6 for the GAPDH assay. The average intra-plate coefficient of variation of the Ct values was 0.74 for the telomere assay and 0.47 for the GAPDH assay. The average Ct for the telomere and GAPDH assays were 12.44 and 27.08 respectively.

Telomere length measurements were calculated using the method by Pfaffl (2001). The mean values were used to calculate the telomere length (T:S ratio) using the formula: ((1 + E telomere)^ ΔCq telomere (control – sample) / (1 + E GAPDH)^ ΔCq GAPDH (control – sample)).In addition, we had sufficient DNA from a small number of 11 day samples (5) that enabled us to measure absolute telomere length using the in-gel TRF method (see Nussey et al., 2014 for details of this method). This enabled us to compare telomere length in wild and captive zebra finches.

Statistical analysis

All statistical analyses were performed in R 4.0.3 (R Core Team, 2017). Linear mixed models (LMMs) were run using the package lmer (Bates, Machler, Bolker, & Walker, 2015), with package lmerTest (Kuznetsova, Brockhoff, & Christensen 2016) to calculate degrees of freedom and *p*-values. We calculated marginal R^2 values for the LMMs using the method described by Nakagawa and Schielzeth (2013). We initially performed a series of linear regression analyses to test for possible effects of body size on telomere length, possible effects of body size on percent DNAm changes and possible effects of temperature on growth rate/body size (Supplementary Analysis 1). We also performed a LMM to examine the effect of baseline telomere length (at day 3) on change in telomere length after adjusting for the effects of regression to the mean (RTM) (Verhulst, Aviv, Benetos, Berenson, & Kark, 2013; Supplementary Analysis 2).

In a first, longitudinal analysis, we used a LMM -Model 1 (sample size 24 individuals) to explore the effects of temperature, clutch size, change in DNAm and change in tarsus (growth

from day 3 to day 11) (all as fixed effects) on the rate of changes in telomere length from day 3 to day 11 (dependent variable). Our preliminary analysis (Supplementary analysis 2c) detected a negative correlation between tarsus length at day 3 and telomere length at day 3. Therefore, we also included tarsus length at day 3 as a fixed effect to account for potential effects of body size on telomere length changes. Nest ID was included as a random effect in this model to control for possible familial/genetic effects, and the repeated sampling of the same individuals within a brood. Only individuals with data for both day 3 and day 11 were included in this longitudinal analysis. Change between day 3 and day 11 was calculated using the equation (*change = (value at day 11 – value at day 3))*. Models were built on the biological variables of interest, and a power analysis was conducted using the R package SIMR in to assess whether our sample size was large enough to test detect statistically meaningful effects (Green and MacLeod, 2015).

In our cross-sectional analyses, we performed two LMMs to examine a) Model 2 (sample size, 33 individuals): the effects of temperature, clutch size, DNAm at day 3, and tarsus length at day 3 (fixed effects) on telomere length at day 3 (the dependent variable), and b) Model 3 (sample size, 28 individuals): the effects of temperature, brood size, DNAm at day 11, and tarsus length at day 11 (fixed effects) on telomere length at day 11. We accounted for genetic and/or familial effects by including Nest ID as a random effect.

RESULTS

The telomere length of the five wild zebra finches measured using the in-gel TRF method was similar to that of captive bred birds (range 12.2-13.59kb, mean 12.77kb+/-0.24). Overall, there was no change in telomere length between day 3 and day 11 of post-hatch development, however, on average mean telomere length was 0.84 (range: 0.29-2.59, SEM: 0.059) on day 3, and 0.74 (range: 0.44-2.16, SEM: 0.041) on day 11. This decrease in telomere length was not statistically significant (paired t-test: t_{49} = 1.59, p= 0.116) (Figure 1). Thirty-three individuals showed a reduction in telomere length between day 3 and day 11 post-hatch, while 17 were found to have telomeres that increased in length. Telomere length decreased by an average of 0.28 (range: -0.64 to -0.02, SEM: 0.03), and increased by 0.25 (range: <0.01 to 1.92, SEM: 0.16).

Our MS-AFLP analyses verified 92 consistent CpG loci between 50 and 500 base pairs. DNAm averaged 29.2% at day 3 (Standard deviation (SD) = 6.5), and 34.9% at day 11 (SD=8.3). Overall, DNAm declined significantly between day 3 and day 11 of post-hatch development

(paired t-test: t_{44} = -6.28, p= <0.001). In our first LMM (n = 24 individuals) we found a significant, negative relationship between changes in the level (%) of DNAm across these 92 CpG loci and change in telomere length between day 3 and day 11 (Table 1, Figure 2a). We also found a significant, negative relationship between change in tarsus length and change in telomere length between day 3 and day 11 (Table 1, Figure 2b). Tarsus length at day 3, brood size and post-hatch ambient temperature did not significantly affect change in telomere length (Table 1). The fixed factors explained 43.73% (Marginal R^2=0.437), while the random effect of natal nest ID explained <1% of the variance in the data (Conditional R^2=0.441; this value describes the proportion of variance explained by both the fixed and the random factor, thus most variance in this model was explained by the fixed factors) (Table 1)). Our power analysis suggested that a sample size of n >=16 was required to detect an effect of our fixed factors on telomere length change with 80% power at a 0.05 significance level, thus our sample size (n= 24) was likely sufficient.

In our second LMM (n= 33 individuals) we detected a significant, positive association between clutch size and telomere length at day 3 (i.e., larger clutches were associated with longer telomeres at day 3 (Table 2)), while percent DNAm at day 3 and other fixed factors (pre-hatch ambient temperature and tarsus length at day 3) were not related to telomere length. In this model, the fixed factors explained 28.57% of the variance in telomere length at day 3 (Marginal R^2=0.285), while the random effect of natal nest ID explained 40.19% of variance in telomere length at day 3 (Conditional R^2=0.687) (Table 2).

In our third LMM (n = 28 individuals) DNAm at day 11 was not related to telomere length at day 11, and, similarly none of the other fixed factors (brood size, temperature and tarsus length at day 11) included in our model appeared to influence telomere length at day 11 (Table 3). The fixed factors in our model (Table 3), explained 8.63% of the variance in the data (Marginal R^2=0.086), while the random effect of natal nest ID explained 59.43% of variance in telomere length at day 11 post-hatch (Conditional R^2=0.6807) (Table 3). After visual inspection of figures 1, 2a and 2b, we identified an individual with a particularly high telomere length value. We re-ran all our models after excluding this anomalous data point, however, excluding this data point did not alter our results significantly. Since there was no biological reason to omit this individual from the data set it was included in our final analyses.

DISCUSSION

Our study investigated the relationship between telomere length and DNAm dynamics in wild animals exposed to naturally variable early life conditions. In our longitudinal analysis we found a negative relationship between individual changes in telomere length and changes in the level of methylated CpG loci (92 CpG loci in our study) across early life, such that individuals with accelerated rates of telomere shortening were associated with increases in DNAm levels. The observational nature of our field data means that we were unable to establish whether the negative relationship between telomere length and DNAm changes reflected coincidental or causal effects. That is, whether DNAm and telomere length responded comparably to the same intrinsic/extrinsic conditions experienced across early life and/or whether potential regulatory effects between DNAm and telomere length occurred. How changes in telomere length and DNAm relate to each other and respond to early life conditions warrants further investigation since it may help identify potentially distinct aspects of biological age that DNAm and telomere length reflect. It is important to note that while variation in DNAm levels may reflect variation in the early life environment (Angers, Castonguay, & Massicotte, 2010; Sheldon et al., 2018; 2020; Makinen, van Oers, Eeva, Laine & Ruuskanen, 2020), the relevance of early-life hypo or hyper methylation in the context of biological age remains unclear. Thus, while our MS-AFLP analyses are useful to compare DNAm and telomere length associations in different environments, next-generation sequencing data (used to generate an 'epigenetic clock') are necessary to compare DNAm and telomere length predictors of biological age in different environments (Horvath & Raj 2018; Banszerus et al., 2019). Furthermore, it is important to note that the DNAm levels derived using blood samples in our study may not be representative of all tissues (Siller and Rubenstein, 2019; Husby, 2020; Schultz et al., 2015). However, several studies have shown correlations between methylation patterns in the blood and other tissues (Masliah et al., 2013, Farré et al., 2014). Using blood samples has successfully revealed links between epigenetic variation and phenotypic variation in other species (primarily humans) (Groleau et al., 2014; Puglia et al., 2015; Ursini et al., 2011; Provencal et al., 2014), and has been informative in describing the link between the environment and the genome. Further, and perhaps more importantly for our study, the use of blood allowed repeat sampling of the same individual across early life, enabling *changes* in DNAm and telomere length to be assessed, which may better link early life conditions and fitness prospects than cross-sectional measures alone (Boonekamp, et al., 2014; Wood & Young, 2019).

In our study, we did not detect a relationship between cross-sectional measures of DNAm and telomere length at either day 3 or day 11 post-hatch. However, telomere length at day 3 was

weakly, but significantly positively associated with clutch size. We have previously shown that DNAm was affected by brood size during early life of wild zebra finches (Sheldon et al., 2018), and experimental work has also shown that telomeres are generally shorter when brood size is increased during early life (Voillemot *et al.,* 2012; Reichert et al., 2014), potentially as a response to the stress of lower per capita food delivery rates to offspring (Costanzo et al., 2016). The positive relationship detected in our analysis is thus interesting and may reflect higher quality parental care (Bichet et al., 2020), lower rates of nestling/egg mortality, or optimal environmental factors among chicks developing in larger clutches (Van Noordwijk & de Jong, 1986). Alternatively, the positive relationship could be due to parental age if older parents had a reduced clutch size and their offspring inherited shorter telomere lengths or lost more up to this point (Monaghan and Metcalfe, 2019). The positive relationship between telomere length and clutch size also suggests that the within clutch decline in telomere length across the ovulation order that has been described in a study on captive zebra finches (Noguera et al., 2020) did not have much effect here.

Evidence is accumulating to suggest that longitudinal measures of within-individual change in telomere length may represent a more useful biomarker of ageing/individual condition than static, cross-sectional telomere length measures (Wood and Young, 2019; Boonekamp et al., 2014; Tricola et al., 2018; Wilbourn et al., 2018). However, to our knowledge, no other study has conducted longitudinal analyses on the relationship between within-individual change in telomere length and DNAm across early development, in any species. Although it may be expected that factors associated with telomere length would also be associated with telomere length *changes*, telomere length at a given timepoint is the outcome of both initial telomere length and subsequent attrition/elongation, which may not be associated. Indeed, in our supplementary analysis (Supplementary Analysis 2) the initial length of telomeres (at day 3) did not affect the rate of telomere length change across early life after controlling for effects of regression to the mean (Verhulst et al., 2013) (however, other studies have detected this trend (Salomons et al., 2009; Aviv et al., 2009)). Hence, variation in initial telomere length, which may be more influenced by heritable genetic factors (Broer et al., 2013, but see Voillemot et al., 2012), may confound the relationship between environmental factors associated with telomere length versus telomere length *change*, and indeed, may confound the accuracy of telomere length as a biomarker of individual condition later in life (Boonekampp et al., 2014; Wood & Young 2019). In line with this, in our study we found that telomere length *change* was better described by DNAm changes and growth

rate, factors that are extremely sensitive to the environment, while genetic and/or familial effects (represented by nest ID in our models) explained the greatest amount of variance in our cross-sectional telomere length measures at both day 3 and day 11. That said, the high amount of variance in telomere length explained by nest ID was somewhat surprising given the low number of replicates within each nest. Future work should aim to increase the number of siblings analysed to more confidently disentangle environmental vs genetic effects on telomere dynamics.

In our longitudinal analyses, we also detected a significant, negative relationship between tarsus growth and change in telomere length, such that individuals that grew faster lost more telomere length across the measured period of their early life. This relationship has been detected in previous studies, and could be due to increases in cell division, energy expenditure and oxidative stress associated with increased growth rate (Monaghan & Ozanne, 2018). The relationship between telomere length and tarsus growth occurred independently of temperature in our study. However, previous studies have shown that embryonic growth rate, manipulated by small variations (+-1^{o}C) in incubation temperature, negatively affect telomere length in the laboratory (Stier et al., 2020). We did not detect a relationship between cross-sectional measures of telomere length and tarsus length in our attempt to explore proximate effects within individuals. However, in a study examining the same relationship (between telomere length and tarsus length) across generations in a selection experiment on body size in the house sparrow, a relationship was detected (Ringsby et al., 2015).

Contrary to other studies (Stier et al., 2020; Fitzpatrick et al., 2019), we did not find an effect of ambient temperature on telomere length or telomere length changes across development. Temperature has been shown to effect within individual changes in DNAm (Sheldon et al., 2020) and telomere length (Stier et al 2020; Fitzpatrick et al 2019) however, it is unclear if temperature effects these DNA modifications comparably. The temperatures (average daily maximum of 27.6^{o}C) in our study may not have been 'stressful' enough to impact telomere dynamics, and indeed temperature has not affected telomere length in studies on other taxa (McLennan *et al.,* 2018; Boonekamp et al, 2020).

DNAm was found to increase between day 3 and 11 post hatch, in concordance with results from a study on early life DNAm patterns in a different passerine bird (Watson et al., 2019). Although most individuals showed a decrease in telomere length across post-hatch development in our study, this trend was not significant. We attribute increases in telomere length to measurement error combined with the relatively short time interval between measurements (see

e.g., Steenstrup; Hjelmborg; Kark; Christensen & Aviv, 2013) however, Hoelzl; Cornils; Smith; Moodley and Ruf, (2016) and Hoelzl et al., (2016) do provide convincing evidence of telomere elongation in a hibernating rodent. The extent to which mechanisms underlying telomere length elongation may weaken the utility of within-individual telomere length variation and, indeed, the utility of telomere length as a biomarker of biological ageing, is unclear, as telomere repair by telomerase may itself be inhibited by the same factors effecting telomere length (e.g., oxidative damage; Ahmed et al. 2008).

Our measurements of absolute telomere length in wild zebra finches gave telomere lengths similar to those in captive birds measured by the same in-gel TRF method; measurements in the same lab and using the same equipment and protocol for laboratory birds (varying in age from 7 to 120 days) ranged from 10.1 to 16.35kb (Millet & Salmon, pers comm). These data suggest that, in contrast to the laboratory mouse which typically has telomeres several times longer than its wild counterparts possibly due to inbreeding (Manning et al 2002), but this is not the case for the zebra finch.

In conclusion, our study of wild zebra finches has confirmed previously documented effects in captive zebra finches of both growth rate and clutch size on telomere length dynamics during early life. This consistency is important given the differences in resources and variability in the environment of wild compared to captive animals. Most significantly, our study detected a negative relationship between early life telomere and DNAm dynamics, such that individuals with accelerated rates of telomere shortening have associated increases in DNAm levels. The nature of this relationship warrants further investigation since it may shed light on potentially clinically/ecologically distinct aspects of biological age reflected by DNAm and telomere length. It would be valuable for future work to focus on next-generation sequencing techniques (e.g., reduced representative bisulfite sequencing (RRBS) or enzymatic-methyl sequencing) to compare DNAm and telomere length measures of biological age across different environments and/or to compare potentially functional relevant or regulatory associations between DNAm and telomere length across different environments. Our results provide a base for further investigations to link and/or disentangle the relationship between the early life environment and DNA biomarkers of biological age.

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DATA ACCESSIBILITY

Authors have archived their data in the publicly accessible repository FigShare. DOI: 10.6084/m9.figshare.13670044

STATEMENT OF CONFLICTING INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

E.L.S., P.M. and S.C.G. conceived the study. E.L.S. collected the field data. E.L.S. and A.W.S. performed the MS-AFLP assay. W.B., R.T., S.R., and P.M. performed the qPCR assay. E.L.S. and R.T. conducted the statistical analyses. The manuscript was written by E.L.S. and commented on and edited by all authors. S.C.G provided funding.

AUTHOR ORCID I.D.

Elizabeth L. Sheldon https://orcid.org/0000-0001-5002-3006
Riccardo Ton https://orcid.org/0000-0003-3897-9720
Simon C. Griffith https://orcid.org/0000-0001-7612-4999
Aaron. W. Schrey https://orcid.org/0000-0002-2165-7349
Pat Monaghan https://orcid.org/0000-0003-2430-0326;
Winnie Boner https://orcid.org/0000-0003-1702-4298
Shirley Raveh https://orcid.org/0000-0003-0406-4928

TABLES AND FIGURES

Figure 1. Paired box plot illustrating individual changes in telomere length across early life (from day 3 to day 11 post-hatch) for 50 individuals from 38 broods. Grey lines connect individuals across development. The box plots show the median, lower, and upper quartile of telomere length data.

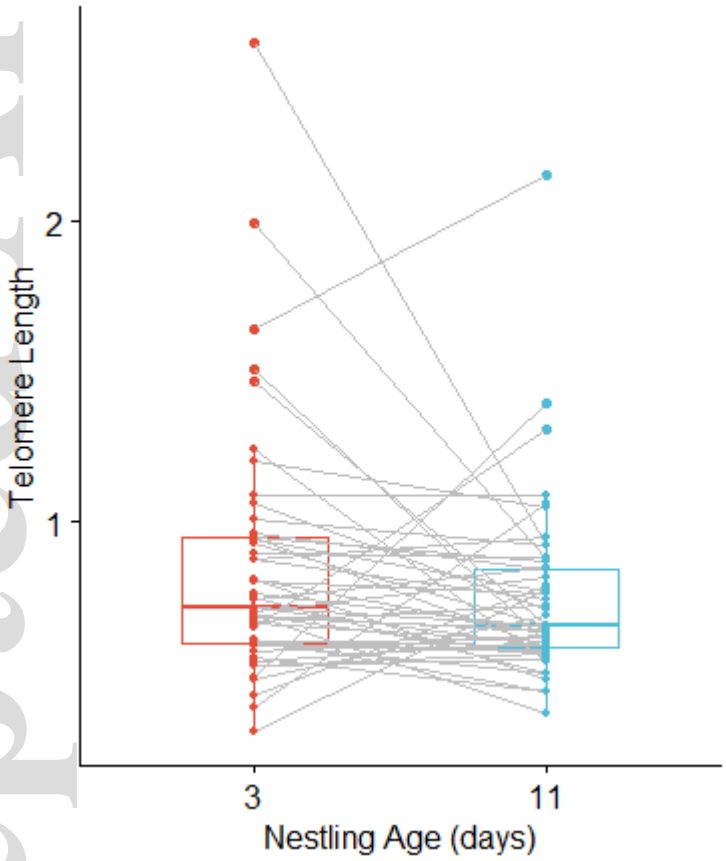

Table 1: Random and fixed effect estimates from a linear mixed model for change in telomere length (n = 24 individuals from 20 nests) from day 3 to day 11 post-hatch. Significant values are indicated by * at the 95% confidence interval. SE = standard error.

Model 1. Dependent Variable: Early life change in telomere length				
Fixed effect	**Estimate**	**SE**	**t-value**	***p*-value**
Change in Tarsus Length	-1.499	0.580	-2.397	0.021*
Change in DNA methylation (%)	-0.875	0.397	-2.126	0.044*
Post-hatch Temperature	0.037	0.026	0.732	0.255
Tarsus Length (day 3)	0.388	0.264	1.469	0.164
Brood Size	>0.001	0.083	-0.566	0.999
Random effect	**Variance**	**St Dev**		
Nest ID	0.002	0.042		
Residual	0.263	0.513		

Figure 2a. Partial residual plot illustrating the relationship between changes in DNA methylation levels (%) (x-axis) and telomere length (y-axis) from day 3-11 post-hatch while controlling for differences in tarsus growth, post-hatch temperature, and brood size.

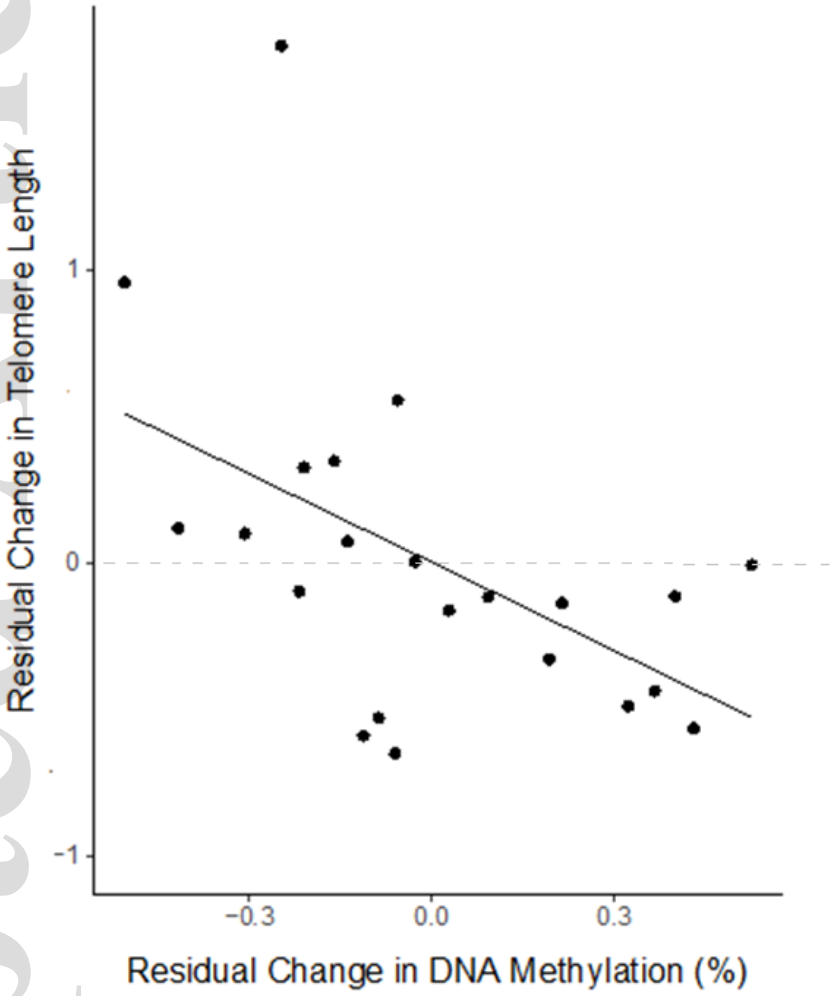

Figure 2b. Partial residual plot illustrating the relationship between tarsus growth (x-axis) and telomere length change (y-axis) from day 3-11 post-hatch while controlling for changes in DNA methylation levels (%), post-hatch temperature, and brood size.

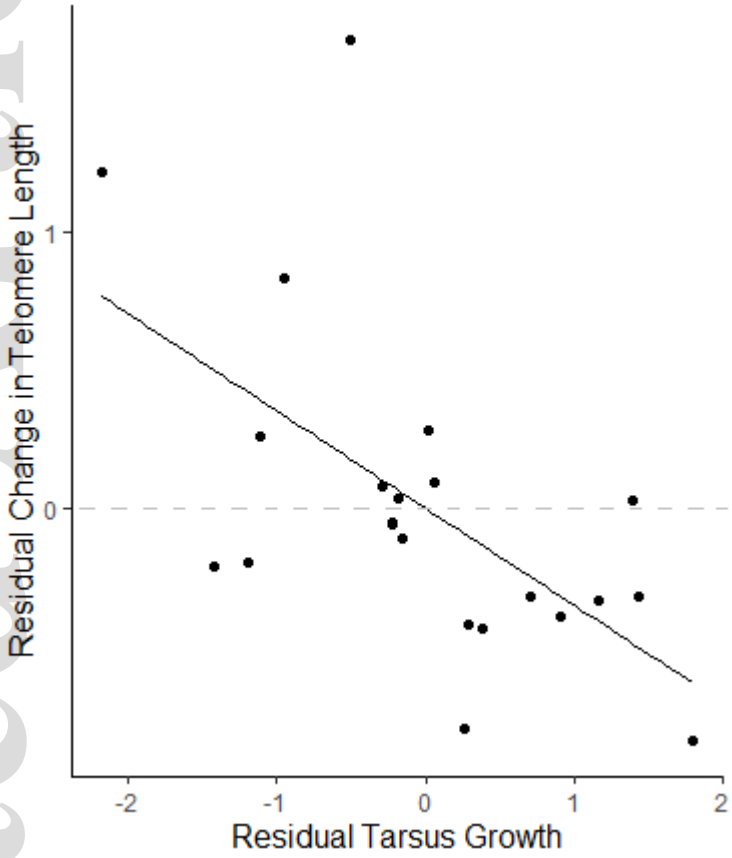

Table 2. Random and fixed effect estimates from a linear mixed model for telomere length at day 3 (n= 33 individuals from 26 nests). Significant values are indicated by * at the 95% confidence interval. SE= Standard error.

Model 3. Dependent Variable: Telomere length at day 3				
Fixed effect	**Estimate**	**SE**	**t-value**	***p*-value**
Tarsus Length (day 3)	-0.132	0.113	-1.168	0.253
DNA methylation (%) (day 3)	-1.787	1.105	-1.617	0.117
Incubation Temperature	0.014	0.020	0.678	0.503
Clutch Size	0.104	0.114	2.275	0.032*
Random effect	**Variance**	**St Dev**		
Nest ID	0.093	0.301		
Residual	0.048	0.221		

Table 3. Random and fixed effect estimates from a linear mixed model for telomere length at day 11 (n = 28 individuals from 23 nests). Significant values are indicated by * at the 95% confidence interval. SE= Standard error.

Model 1. Dependent Variable: Telomere length at day 11				
Fixed effect	**Estimate**	**SE**	**t-value**	***p*-value**
Tarsus Length (day 11)	0.001	0.077	0.017	0.987
DNA methylation (%) (day 11)	0.080	0.786	0.102	0.920
Post-hatch Temperature	0.022	0.016	1.337	0.197
Brood Size	-0.031	0.053	-0.589	0.564
Random effect	**Variance**	**St Dev**		
Nest ID	0.091	0.301		
Residual	0.048	0.221		