Early-life telomere length predicts survival to adulthood and lifespan in a wild mammal

Sil H.J. van Lieshout[1,2], Amanda Bretman[1], Chris Newman[3], Christina D. Buesching[3], David W. Macdonald[3] & Hannah L. Dugdale[1]

[1]School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK; [2]NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK; [3]Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, The Recanati-Kaplan Centre, Abingdon, Oxfordshire OX13 5QL, UK

Correspondence author: Sil H.J. van Lieshout

E-mail: sil.vanlieshout@gmail.com

ORCID: SHJvL, 0000-0003-4136-265X; AB, 0000-0002-4421-3337; CN, 0000-0002-9284-6526; CDB, 0000-0002-4207-5196; DWM, 0000-0003-0607-9373 ; HLD, 0000-0001-8769-0099

Abstract

Telomeres, protective caps at the end of chromosomes, maintain genomic stability and function as a biomarker of senescence in many vertebrate species. Telomere length at different ages has been related to (subsequent) lifespan, but to date only one laboratory-based study has shown a direct link between early-life telomere length and lifespan. Whether this relationship holds in wild populations, where individuals are subject to variable natural conditions that may mask relationships seen in controlled laboratory settings, remains unknown. Here, we provide evidence that early-life telomere length predicts survival to adulthood in a wild population of European badgers (*Meles meles*). Furthermore, both early-life telomere length and telomere rate of change predict lifespan. We found a complex cross-sectional relationship between telomere length and age, where telomeres shortened over the first 38 months, but with no uniform loss after this point. We found little within-individual consistency in telomere length across lifespan, where telomere length did not predict residual lifespan.

Importantly, we also observed increases in mean telomere length within individuals, which could not be explained by measurement error alone. Early-life telomere length varied distinctly among cohorts, indicating a role for early-life environment and additive genetic effects. Our results elaborate on the dynamic way that telomeres function as a biomarker of senescence in a wild mammal, where telomere length and rate of change can reflect short-term and lasting effects of early-life conditions on individual life-history.

Keywords: telomere length, early-life conditions, biomarker, senescence, wild population, mammal

1. Introduction

Species from most taxa exhibit a loss of performance with age that leads to a greater probability of mortality (Medawar 1952; Williams 1957). This process of senescence is common, but highly variable across taxa (Jones *et al.* 2014) and even within species (Campbell *et al.* 2017; Dugdale *et al.* 2011; Nussey *et al.* 2009). Pioneering laboratory studies using controlled environments have provided important insights into senescence patterns, but cannot explain the remarkable variation in the onset and rate of senescence in wild populations, where selection acts under naturally varying conditions (Partridge & Gems 2007). It has also proven challenging to test how the natural environment drives variation among individual senescence patterns, and thus consequences for individual mortality risk cannot be determined easily. A biomarker, such as telomere length (Monaghan & Haussmann 2006), that reflects the physiological consequences of within-individual experiences and facilitates between-individual comparisons, is therefore valuable.

Telomeres are non-coding hexameric repeats (5'-TTAGGG-3') that, with associated shelterin proteins, prevent end-to-end fusion of linear chromosomes and maintain genomic integrity (Blackburn 2000; de Lange 2004). Telomeres shorten with age due to incomplete DNA-replication at the 3'-end of the DNA-strand (Olovnikov 1973). This occurs more rapidly in early-life due to higher levels of cellular

division during growth (Frenck *et al.* 1998; Hall *et al.* 2004), or in response to metabolically demanding activities (e.g. reproduction; Heidinger *et al.* 2012; coping with stress/disease; Epel *et al.* 2004; Willeit *et al.* 2010). The amount of telomeric DNA lost in each cell division depends on cellular conditions (Monaghan & Ozanne 2018) and oxidative stress (Reichert & Stier 2017; von Zglinicki 2002; but see Boonekamp 2017). Telomeres can, however, be replenished by telomerase, the telomere-elongating enzyme (Blackburn *et al.* 1989). Telomerase is transcriptionally repressed later in development (Blackburn *et al.* 1989), but alternative pathways for telomere lengthening do exist (Cesare & Reddel 2010; Mendez-Bermudez *et al.* 2012). Telomere erosion occurs until cells enter a state of arrest, inducing replicative senescence, where the accumulation of senescent cells, due to progressive loss of regenerative capacity (Campisi & di Fagagna 2007), can impair tissue functioning (Armanios & Blackburn 2012; Campisi 2005).

In humans, mean leukocyte telomere length (LTL) shows a biphasic decline with age, although inter-individual variation exists (Monaghan 2010), with rapid shortening in early-life followed by slower attrition in adulthood (Aubert & Lansdorp 2008). Variation in LTL has been linked to variation in survival probability, where a cross-sectional study reported that people with longer-than-average telomeres have higher survival probabilities than individuals with shorter-than-average telomeres (Cawthon *et al.* 2003). In birds, associations between erythrocyte telomere length (ETL) and survival probability have been found (Barrett *et al.* 2013; Bize *et al.* 2009) and recently relationships between LTL and survival probability have been revealed in wild mammals (Cram *et al.* 2017; Fairlie *et al.* 2016). Moreover, a meta-analysis of non-human vertebrates reported an overall association between telomere length and survival probability (Wilbourn *et al.* 2018). These relationships indicate that telomere length can represent short-term effects, particularly when within-individual telomere length can both increase and decrease with age (Fairlie *et al.* 2016; Spurgin *et al.* 2017). Furthermore, depending on the amount of telomere elongation relative to the overall telomere shortening, telomere length could also reflect long-term effects.

Studies finding short-term effects are often cross-sectional, where senescence patterns can be masked by, for example, selective disappearance or cohort effects (Nussey *et al.* 2008). These studies only sample individuals at a specific life-stage, so the individuals' preceding and subsequent telomere lengths remain unknown, preventing determination of variation in rate of change. Consequently, longitudinal studies, which can account for population-level effects, are required to determine lifelong variation in telomere length and potentially long-term effects. For example, as variation in telomere shortening might be related to (maximum) lifespan (Tricola *et al.* 2018) and survival probability (Boonekamp *et al.* 2014; Salomons *et al.* 2009), repeated measurements per individual are needed, requiring longitudinal studies. While such studies remain relatively rare in mammals (Beirne *et al.* 2014; Fairlie *et al.* 2016), one longitudinal laboratory-based avian study found a long-term effect where early-life telomere length predicted lifespan (Heidinger *et al.* 2012).

Early-life telomere length, shaped by early-life conditions such as maternal effects, developmental stress and competition (e.g. Asghar *et al.* 2015; Haussmann *et al.* 2012; Cram *et al.* 2017), can be particularly important. This is because natural selection acts on the proportion of a cohort that is alive, which is greatest in early-life (Hamilton 1966). Moreover, individuals often differ substantially in early-life telomere length (Fairlie *et al.* 2016). This variation in early-life telomere length can be due to additive genetic effects (Asghar *et al.* 2015; Becker *et al.* 2015; Dugdale & Richardson 2018) and may be impacted strongly by the early-life environment (Boonekamp *et al.* 2014; Cram *et al.* 2017; Nettle *et al.* 2015). Early-life telomere length, during this sensitive period, may therefore be a better predictor of lifespan than when measured across a wider range of ages. However, whether the relationship between early-life telomere length and lifespan holds in wild populations currently remains unknown.

The European badger (*Meles meles*; henceforth 'badger') provides an informative mammalian model species for studying the effects of early-life conditions on telomere length and senescence patterns. We benefit here from a long-term study of badgers at Wytham Woods (Oxford, UK;

Macdonald *et al.* 2015); an almost closed population (see Macdonald *et al.* 2008) with a high and relatively consistent annual recapture rate of 84% (SE = 1.3%; Macdonald *et al.* 2009) over 1726 life-histories monitored seasonally since 1987. In this population, badgers live in polygynandrous social groups (mean group size: 11.3, range: 2 – 29; da Silva *et al.* 1994; Macdonald *et al.* 2015), and senesce (Dugdale *et al.* 2011). First year survival probability ranges from 61 – 94% (mean ± SE = 0.67 ± 0.03; Macdonald *et al.* 2009), and cub cohorts are impacted by early-life exposure to endo-parasitic coccidia infection (Newman *et al.* 2001), oxidative stress (Bilham *et al.* 2018) and unseasonable weather variation (Macdonald *et al.* 2010; Noonan *et al.* 2014; Nouvellet *et al.* 2013). We therefore posit that strong selection pressures on badger cubs may be reflected in their telomeres, enabling us to test for a relationship between telomere length and lifespan.

We determined badger telomere lengths by re-sampling individuals longitudinally. As relative leukocyte telomere length (RLTL) is likely to change most rapidly in early-life (a consequence of faster cell replication associated with growth), we first needed to account for age in statistical models. We therefore tested the hypothesis that RLTL is affected by age, predicting a negative relationship with shorter RLTL at older ages. Secondly, consistent with recent findings in wild populations, we hypothesised that RLTL may vary substantially across an individual's lifespan, including periods of telomere elongation as well as overall attrition, which would be evidenced by low individual repeatability in RLTL. Thirdly, we determined the relationship between RLTL and lifespan. We therefore tested the hypothesis that early-life RLTL functions as a biomarker for short-term, but not long-term effects, and that RLTL does not predict years to death. Support for these hypotheses would come from demonstrating a relationship between early-life RLTL and survival to adulthood (short-term effect), but not lifespan or, when measured at a given point (age), residual lifespan. Finally, we tested whether early-life telomere rate of change (TROC) predicted lifespan and if this depended on early-life RLTL.

2. Methods

2.1 Study system

We conducted this study in Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W), a 424 ha mixed semi-natural woodland site surrounded by mixed arable and permanent pasture (Macdonald & Newman 2002; Macdonald *et al.* 2004; Savill 2010). The resident high-density badger population (range = 20.5 – 49.5 badgers/km^2; Macdonald *et al.* 2015) forms large social groups (Johnson *et al.* 2000). Badger social groups have clearly demarcated territories (Buesching *et al.* 2016; Delahay *et al.* 2000), although badgers do cross these borders when foraging and meet amicably with neighbouring groups (Ellwood *et al.* 2017; Noonan *et al.* 2015). Mean annual adult survival rates in this population are 0.83 (± 0.01 SE, Macdonald *et al.* 2009).

Trapping has been undertaken three or four times per year since 1987, for two to three consecutive days per social group. Trapped badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren *et al.* 2005) and identified by a unique tattoo number on the left inguinal region. Capture date, sett, social group (comprising several setts, i.e. burrow systems), sex, age-class (cub <1 year; adult ≥1 year) and morphometric measurements (i.e. length, weight, tooth wear; da Silva & Macdonald 1989; Macdonald *et al.* 2009) were recorded for each badger. The age of a badger was defined as the number of days elapsed since the 14th of February in their respective birth year (reflecting the February birth peak; Yamaguchi *et al.* 2006). Blood was collected by jugular venipuncture into vacutainers with either an EDTA or Heparin anticoagulant and stored at -20°C immediately. Badgers were released at their setts, after full recovery from anaesthesia.

2.2 Telomere analyses

We selected 1248 blood samples from 612 individuals, representing 308 males and 304 females, comprising individuals varying in lifespan (range: 14 – 233 months; mean ± SE = 97.2 ± 1.88 months). Only badgers for which age could be determined, either trapped as cub (*n* = 545) or inferred through

low tooth wear were included (*n* = 67, males = 26, females = 41; i.e. tooth wear 1 indicates a cub and tooth wear 2 indicates a yearling (da Silva & Macdonald 1989; Macdonald *et al.* 2009), where young individuals also had to have length <685 mm and weight <8 kg). Individuals were either sampled once (*n* = 163) or more (*n* = 449 badgers; 2 – 9 times per individual) for telomere length analyses. All analyses were also run without the 67 individuals for which age was determined through tooth wear, to confirm that inclusion of these samples did not bias the results (see supplementary material).

Genomic DNA was extracted from whole blood using the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, with adjustments using 125 µl of anticoagulated blood and a double elution step (2x 75 µl AE buffer). DNA integrity was assessed by running a random selection of DNA extracts (± 20%) on agarose gels to check for high molecular weight. DNA concentration was quantified using the Fluostar Optima fluorometer (BMG Labtech, Ortenberg, Germany) and standardized to 20 ng/µl, after which samples were stored at -20 °C until monochrome multiplex real-time quantitative PCR was conducted on the Quantstudio 12K flex real-time PCR system (Applied Biosystems, Warrington, UK; a detailed description of the methodology is provided in the supplementary material).

2.3 Statistical analyses

Statistical analyses were conducted in R 3.3.1 (R Development Core Team 2018), with RLTL measurements square-root transformed to meet the assumptions of Gaussian error distributions in models with RLTL as response variable. Model averaging was conducted, using an information theoretic approach to select plausible models and estimate the relative importance of fixed effects for models with ΔAIC <7 with the "natural average method" (Burnham *et al.* 2011).

2.3.1 Cohort and age effects on telomere length

The relationship between RLTL and age (months), and the interaction with cohort, was assessed following Fairlie *et al.* (2016) and Spurgin *et al.* (2017), testing a variety of age functions (e.g. linear, quadratic, full-factorial, thresholds) in linear mixed models (Bates *et al.* 2015) that controlled for individual ID, plate ID, year and sample storage time. We did not fully apply model selection or averaging, but instead compared a set of specifically defined models, where we considered models with ΔAIC <7. Next, we split the age effect into within- and between-individual effects (van de Pol & Wright 2009) and included age of last reproduction (van de Pol & Verhulst 2006) to test if selective disappearance of individuals contributed to the age pattern observed.

2.3.2 Individual repeatability and telomere elongation

Individual repeatability (across multiple samples from the same individual) was calculated by dividing individual variance by total variance, in a Gaussian-distributed model (identity link function) with RLTL as response variable and age, cohort and individual ID as random effects. Additionally, we determined the correlation between within-individual telomere measurements, using the marginal R^2 (Nakagawa & Schielzeth 2013), in a Gaussian-distributed model (identity link function) with RLTL as response variable, RLTL at *t+1* and age as fixed effects and cohort and individual ID as random effects.

Increases in RLTL with age were examined by estimating differences in variance in telomere lengths among technical replicates and among within-individual samples using Levene's test following Spurgin *et al.* (2017), to test whether changes in RLTL were greater than measurement error. Further differentiation of samples, grouped according to increasing or decreasing RLTL, were made using Wilcoxon tests to determine whether within-individual change in RLTL was greater than measurement error for these groups separately. Additionally, we tested if the residual error variance ($\sigma_{\varepsilon}^{-2}$) was smaller than the error variance in RLTL, when RLTL can increase or decrease ($\sigma_{\varepsilon}^{'2}$), following Simons *et al.* (2014), which would reject the hypothesis that RLTL shows no elongation.

200 2.3.3 Telomere length and lifespan

201 We used GLMMs to test the relationship between early-life RLTL (<1 year old) and survival to adulthood
202 (*n* = 435), where in all of the following five models we tested for first order and quadratic effects and
203 removed quadratic effects when non-significant. All five models included sex and cohort as fixed
204 effects and plate, natal social group and sample storage time as random effects. First, we modelled
205 survival to adulthood (>1 year old) using a binary term in a binomial (logit link function) mixed-effects
206 model with early-life RLTL and age in months as additional fixed effects. Secondly, early-life RLTL as a
207 predictor of lifespan was modelled with lifespan as response variable, including early-life RLTL and age
208 in months as additional fixed effects in a Poisson-distributed model (log link function). We also
209 controlled for overdispersion by including observation (for each unique measure) as a random effect
210 (Harrison 2014). Lifespan was calculated as the difference between the day of birth and last capture,
211 with 12 months added when last captured as cub and 24 months for adults due to their different
212 survival rates (Macdonald *et al.* 2009) and a 95% recapture interval of 2 years (Dugdale *et al.* 2007).
213 Thirdly, we tested the relationship between RLTL and residual lifespan in a Poisson-distributed model
214 (log link function) with RLTL and age (based on best fitting model) as additional fixed effects and
215 individual ID (correcting for multiple measures per individual), current social group and year as
216 additional random effects. Fourthly, we tested the relationship between early-life telomere rate of
217 change (<3 years old) and lifespan (*n* = 291) in a Poisson-distributed model (log link function). Telomere
218 rate of change was calculated per individual as the slope of a linear regression line for telomere length
219 versus age (Haussmann *et al.* 2003b; Tricola *et al.* 2018), with no indication for a non-linear regression.
220 The model for lifespan contained telomere rate of change, mean early-life RLTL (<3 years old) and
221 mean age per individual as additional fixed effects and year, current social group and observation as
222 additional random effects. Finally, we tested whether individuals with longer-than-average mean
223 early-life RLTL, to avoid regression to the mean, had higher rates of telomere shortening in a Gaussian-

distributed model (identity link function), using mean early-life RLTL (<3 year old) and mean age as additional fixed effects and year as additional random effect.

3. Results

3.1 Cohort and age effects on telomere length

Across all samples, after an initial decrease to 38 months, RLTL increased up to 112 months, followed by a second decrease in RLTL with age (Figure 1). Four models had ΔAIC <7, and all included the threshold at 38 months and a cohort effect (Table S1; Figure 2a and S1). Additional thresholds at 74 or 112 months were only present in two of the four models, and therefore had less support.

Splitting the age effect into within- and between-individual effects per age group showed that within-individual change was more rapid than between-individual change (X^2 = 4.953, P = 0.03) for individuals ≤38 months old but not for other age groups. There was, however, no interaction between mean age and age of last reproduction, indicating that within-individual effects were similar irrespective of age of last reproduction. Selective disappearance of individuals with shorter telomeres therefore does not contribute to the age pattern observed.

Individual repeatability was 0.030 (95% CI = 0.020 – 0.049), so 3% of the variance in RLTL was explained by within-individual consistency among samples. There was no significant correlation between RLTL measured at different time points in the same individual (marginal R^2 = 0.015; X^2 = 2.56, P = 0.110; Figure 2b).

3.2 Telomere elongation

Increases (in the range of 0.004 – 5.829% per month) in RLTL were identified in 56.4% of within-individual changes (Figure 2c) for individuals with ≥2 samples (n = 449). Variance among within-individual samples was higher than variance among technical replicates (Levene's test, $F_{1,1085}$ = 1219.3, P <0.001; Figure 2d) for samples that exhibited an increase in RLTL (Wilcoxon test, P <0.001) and for

249 those that decreased (Wilcoxon test, P <0.001; Figure 2d). Additionally, residual variance among
250 samples was smaller ($\sigma_{\varepsilon}^{-2}$ = 0.018) than the overall change in RLTL ($\sigma_{\varepsilon}^{'2}$ = 0.450; $F_{17,16}$ = 69.12, P <0.001).
251 These positive within-individual changes were therefore not solely due to measurement error.
252
253 3.3 Telomere length, survival and lifespan
254 Early-life RLTL (<1 year old) predicted survival to adulthood (Figure 3 and 4a), but also showed a
255 positive relationship with lifespan (Figure 4b and 5). Individuals with longer telomeres in early-life
256 therefore had longer lifespans, such that an increase of 1 T/S ratio was associated with 8.6% greater
257 longevity. There was, however, no relationship between RLTL, at any given age, and residual lifespan
258 (Figure 4c). Lifespan did show a negative quadratic relationship with TROC (Figure 4d and 6). Individual
259 TROC was not associated with an individual's mean early-life RLTL (β = 0.002, 95% CI = -0.001 – 0.005;
260 Table S2 and Figure S2). All models indicated a strong effect of cohort on individual lifespan (Table S2;
261 Figure 2a and S1).
262
263 **4. Discussion**
264 We found complex telomere dynamics, with both decreases (i.e. in the first 38 months of life and after
265 112 months) and increases in RLTL with age. Splitting this age effect into between- and within-
266 individual effects showed that within-individual change was more rapid than between-individual
267 change in early-life (≤38 months). This was, however, due to within-individual changes, and not
268 attributable to selective disappearance, because it occurred independent of age of last reproduction.
269 Individual repeatability in RLTL was only 3% throughout an individual's lifespan, with no correlation
270 among within-individual RLTL measurements. This was slightly lower than the 7% – 13% range reported
271 in other wild study systems (Fairlie *et al.* 2016; Spurgin *et al.* 2017). However, since most samples in
272 our study were taken early in life, when the greatest telomere changes are expected to occur, a lower
273 repeatability can be expected and so the same individuals do not have consistently longer telomeres.

A greater number of repeated measures, particularly later in life, would likely enhance the individual repeatability estimate, because individuals are more likely to maintain rank due to smaller RLTL changes in later-life. The low individual repeatability indicates that within-individual changes in RLTL are greater than between-individual differences, where positive within-individual changes indicate some active process in maintaining telomere length.

Telomere elongation, particularly in qPCR-based studies, is often attributed to measurement error (Steenstrup *et al.* 2013; Verhulst *et al.* 2015). It is, however, becoming more apparent in wild population studies that telomeres do elongate (Fairlie *et al.* 2016; Hoelzl *et al.* 2016a; Hoelzl *et al.* 2016b; Kotrschal *et al.* 2007; Spurgin *et al.* 2017). Our study is in agreement with this, using monochrome multiplex qPCR that, in principle, reduces measurement error due to reactions occurring in the same well. Additionally, we found that residual variance among samples was smaller than the overall change in RLTL, and variance among technical replicates was smaller than among-sample variation, indicating that increases in mean telomere length with age were not due to measurement error alone. We acknowledge, however, the potential for competing mechanisms that could alter mean RLTL, notably leukocyte cell composition changes with age (Kimura *et al.* 2010; Linton & Dorshkind 2004; Pawelec *et al.* 2010; Weng 2012). Mammalian leukocytes are nucleated and different leukocyte cell types have different telomere lengths due to their respective functional capacities to proliferate and express telomerase (Aubert & Lansdorp 2008; Weng 2001), and vary in ratio over time with health/immune status (see Davis *et al.* 2008). For instance, an innate immune response can cause a granucolyte-biased leukocyte ratio, where granulocytes have longer telomeres than lymphocytes in humans and baboons (Baerlocher *et al.* 2007; Kimura *et al.* 2010). While a previous study of RLTL on wild Soay sheep (*Ovis aries*) did not find changes in leukocyte cell composition with age (Watson *et al.* 2017), leukocyte cell composition in badgers does vary between similar aged cubs and across an individual's lifespan due to changes in immune system activation (McLaren *et al.* 2003). Greater metabolic rate while clearing infection could also modify leukocyte cell composition and potentially

affect mean RLTL directly. For instance, badger cubs are typically infected with coccidia (Newman *et al.* 2001), causing a strong innate immune response and oxidative stress (Bilham *et al.* 2018; Bilham *et al.* 2013). A change in an individual's immunological status, along with age, may therefore alter individual leukocyte cell composition and might contribute to RLTL elongation in this study.

That early-life effects can have both short- and long-term effects on individual life-history is evidenced in our study through early-life RLTL predicting survival probability and lifespan. This is congruent with previous studies reporting that early-life RLTL predicts survival probability in non-human mammals (Cram *et al.* 2017; Fairlie *et al.* 2016) and shows that early-life RLTL not only predicts lifespan in the laboratory (Heidinger *et al.* 2012), but also in a wild population. In early-life, the forces of selection are strongest because: 1) natural selection acts on the more numerous proportion of a cohort that is alive (Hamilton 1966) and 2) rapid somatic growth induces high rates of cell proliferation and individuals exhibit higher levels of cellular stress (e.g. Frenck *et al.* 1998; Haussmann *et al.* 2003a) as, for example, smaller juveniles have higher metabolic rates due to allometric scale laws (and allocate more energy to immune defenses) – causing more rapid cell division (Glazier 2005). In contrast, RLTL at any given time point did not predict residual lifespan. Given the variability of within-individual changes in telomere length, a single point measure provides an unreliable basis to predict an individual's years until death. Early-life RLTL, during the period subject to strong selective forces, should therefore provide a better predictor of (subsequent) lifespan than when measured across ages. Badgers, however, do not emerge from their underground den before 6 – 8 weeks, and are weaned at around 12 weeks (Neal & Cheeseman 1996), where welfare legislation (Protection of Badgers Act, 1992) prohibits trapping between December and May. Consequently, we might miss the strongest effects on RLTL in the first 3 months of life, resulting in weaker relationships. Nevertheless, sampling takes place from 3 months, and during the first year of life when cubs are still rapidly growing, allowing us to detect early-life effects albeit with potentially conservative effect sizes.

Although other studies have reported higher rates of telomere shortening for individuals with longer telomeres (Bize *et al.* 2009; Nordfjall *et al.* 2009), even when overcoming regression to the mean (Salomons *et al.* 2009), we found that individuals with longer RLTL did not show more rapid telomere loss, evidenced by no association between mean early-life RLTL and TROC. Nevertheless, individuals exhibiting less change in RLTL (positive or negative), and therefore lower TROC, had longer lifespans. Shortening of telomere length is detrimental because it increases the risk of replicative senescence, but elongation of RLTL also negatively affected lifespan. Again, elongation could be due to infection driving a change in leukocyte cell composition, as described above. Reduced lifespan with telomere elongation could also indicate a negative effect of telomerase activity, because although telomerase activation can slow down genomic instability arising from dysfunctionally short telomeres (Kim *et al.* 1994), it can also confer immortality to cancer cells (Kim *et al.* 2016; Robin *et al.* 2014), increasing the likelihood of cancer. Cancers are, however, relatively rare in the wild, and larger animals have evolved specific tumour suppression mechanisms (Caulin & Maley 2011; Peto 2015; Risques & Promislow 2018) with, for example, additional p53 pseudogenes (Vazquez *et al.* 2018), indicating a strong selection pressure against carcinogenesis and therefore perhaps against telomere elongation.

The early-life environment clearly exerted a strong effect on early-life RLTL, apparent from the pronounced variation in early-life RLTL we noted among cohorts. The badgers in our study are exposed to variable conditions and have a limited tolerance for, for example, cohort-specific weather conditions (i.e. succeeding better with intermediate levels of rainfall and restricted deviation from the mean temperature resulting in higher cub survival; Nouvellet *et al.* 2013; Macdonald *et al.* 2010) and exposure to diseases (Newman *et al.* 2001). This may explain the variation in early-life telomere length seen in our study system. Similarly, previous studies in birds have shown that higher levels of early-life competition can accelerate telomere shortening (Boonekamp *et al.* 2014; Nettle *et al.* 2015). In mammals, studies on social and ecological effects on telomere dynamics are emerging (Cram *et al.* 2017; Izzo *et al.* 2011; Lewin *et al.* 2015; Watson *et al.* 2017; Wilbourn *et al.* 2017), showing that, for

example, socially dominant spotted hyenas (*Crocuta crocuta*) have longer telomeres (Lewin *et al.* 2015) and that meerkat (*Suricata suricatta*) pups experiencing more intense early-life competition have shorter telomere lengths (Cram *et al.* 2017).

Another contributing factor to the variation in early-life RLTL could be additive genetic effects (Dugdale & Richardson 2018). In wild populations, using the 'animal model', no heritability of telomere length was found in white-throated dippers (*Cinclus cinclus*; Becker *et al.* 2015), and medium heritability (0.35 – 0.48) was found in the great reed warbler (*Acrocephalus arundinaceus*; Asghar *et al.* 2015). Recently, heritability of telomere length has been determined in dairy cattle (0.32 – 0.38; Seeker *et al.* 2018), but, to date, there are no estimates from wild mammalian species.

Our findings demonstrate the dynamic way that telomeres function as a biomarker of senescence in a wild mammal, where telomere length and rate of change can reflect short-term and lasting effects of early-life conditions on individual life-history. Further work specifically quantifying additive genetic effects and how early-life environment conditions impact telomere lengths in wild mammals, will provide insight into the evolution of senescence.

Ethics

All work was approved by the University of Oxford's Animal Welfare and Ethical Review Board, ratified by the University of Leeds, and carried out under Natural England Licenses, currently 2017-27589-SCI-SCI and Home Office Licence (Animals, Scientific Procedures, Act, 1986) PPL: 30/3379.

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Authors' contributions

The study was conceived by S.H.J.v.L, A.B. and H.L.D, and developed by C.N., C.D.B. and D.W.M.; Samples were collected by S.H.J.v.L., C.N., C.D.B., D.W.M. and H.L.D.; S.H.J.v.L. conducted laboratory work and statistical analyses with input from H.L.D.; The paper was written by S.H.J.v.L and all authors critiqued the output for important intellectual content. All authors gave final approval for publication.

Data Accessibility

Data will be deposited in the Dryad Digital Repository upon acceptance.

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Figures

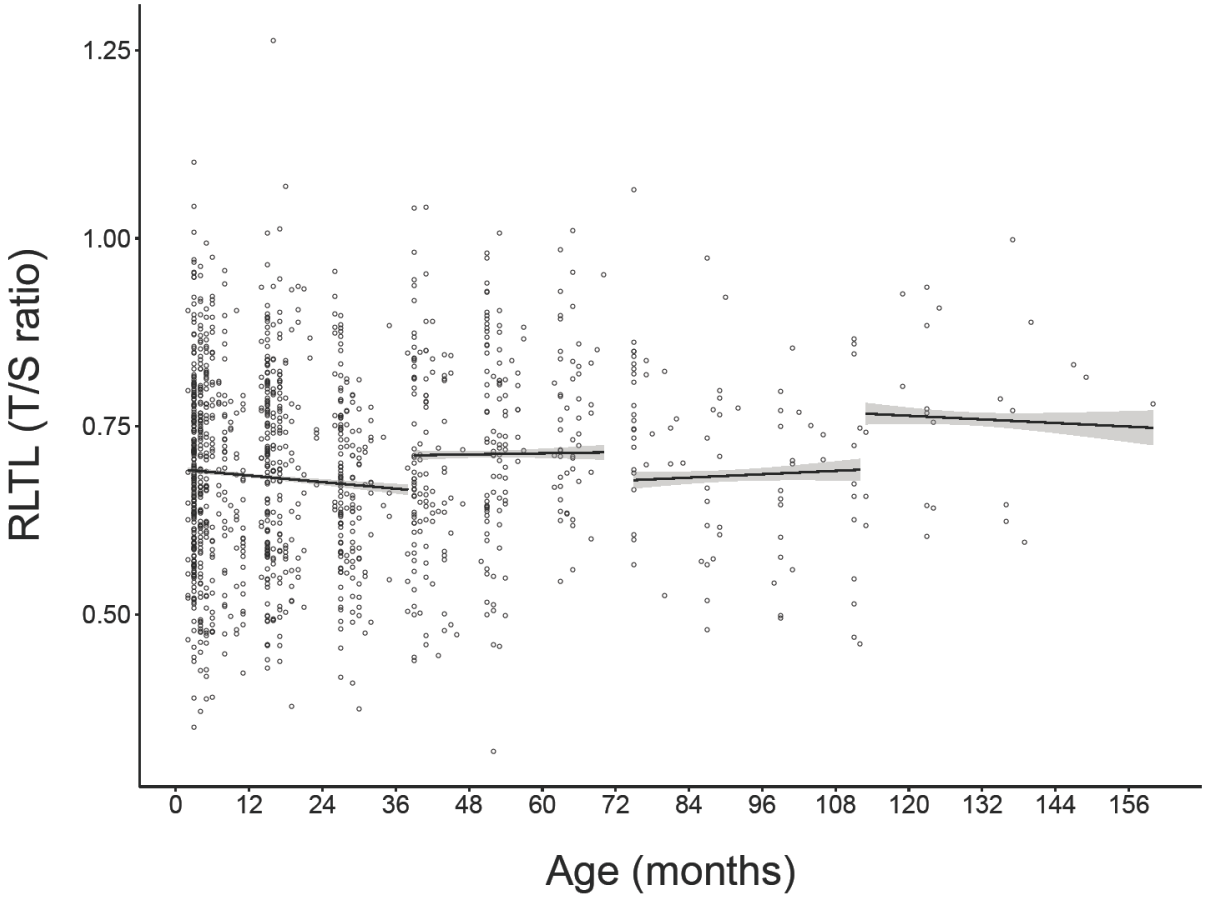

Figure 1: Age-related variation in relative leukocyte telomere length (RLTL), with inflection points at 38, 74 and 112 months old. Raw data points (*n* = 1,248) are shown with fitted lines representing the model prediction for RLTL (T/S ratio) with 95% confidence intervals within the four age groups.

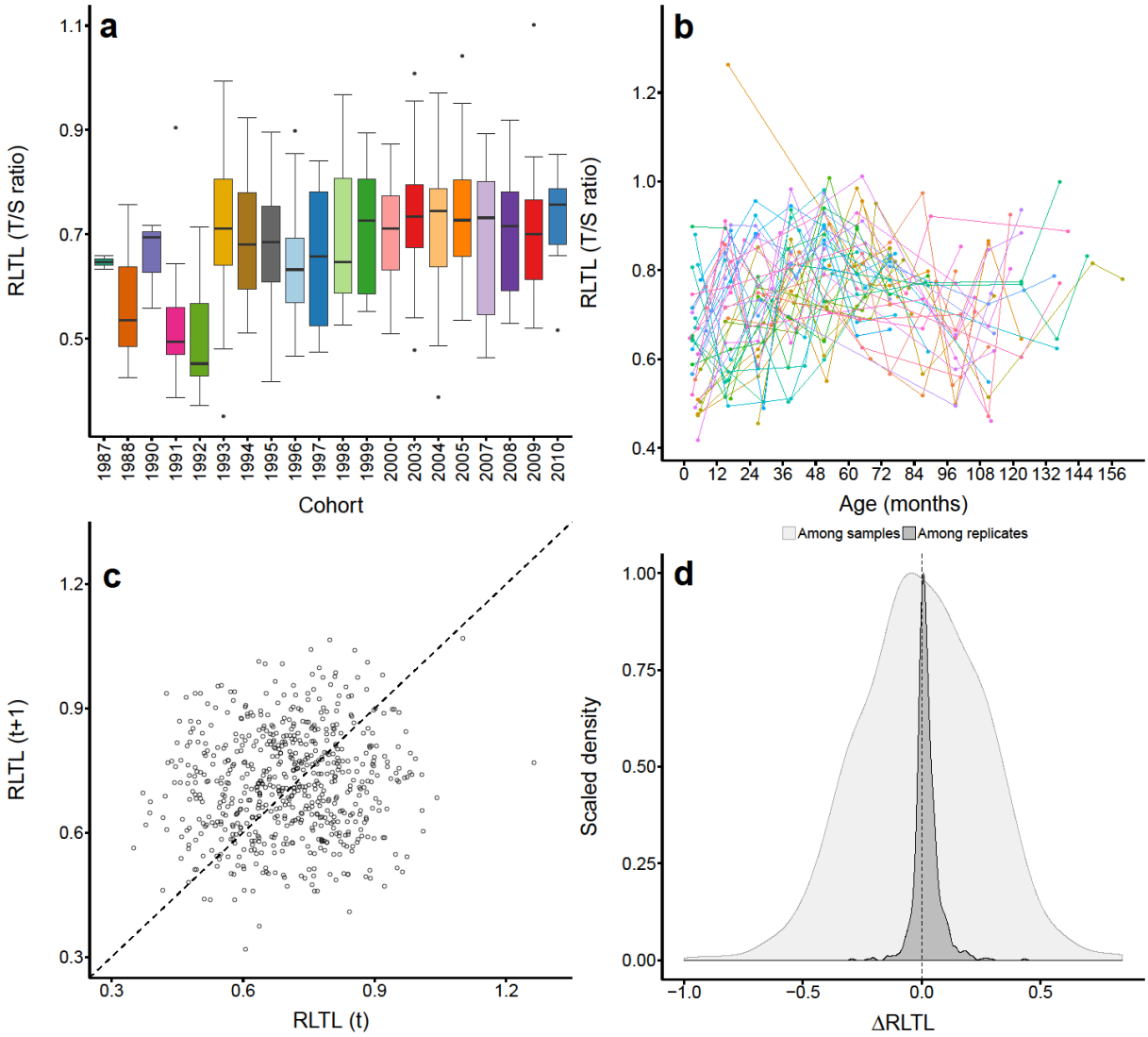

Figure 2: Telomere dynamics in European badgers. a) Variation in early-life relative leukocyte telomere length (RLTL) among cohorts. b) Longitudinal telomere dynamics for 41 individuals that were measured at least four times. c) Within-individual variation in RLTL over consecutive time points (t and t+1). Dashed line represents parity, thus data points above and below this line represent increases and decreases in telomere length, respectively. d) Scaled density plots of changes in RLTL among technical replicates (dark grey) and among individual samples (light grey). Areas left of the dotted line represent decreases in RLTL, while to the right represent increases.

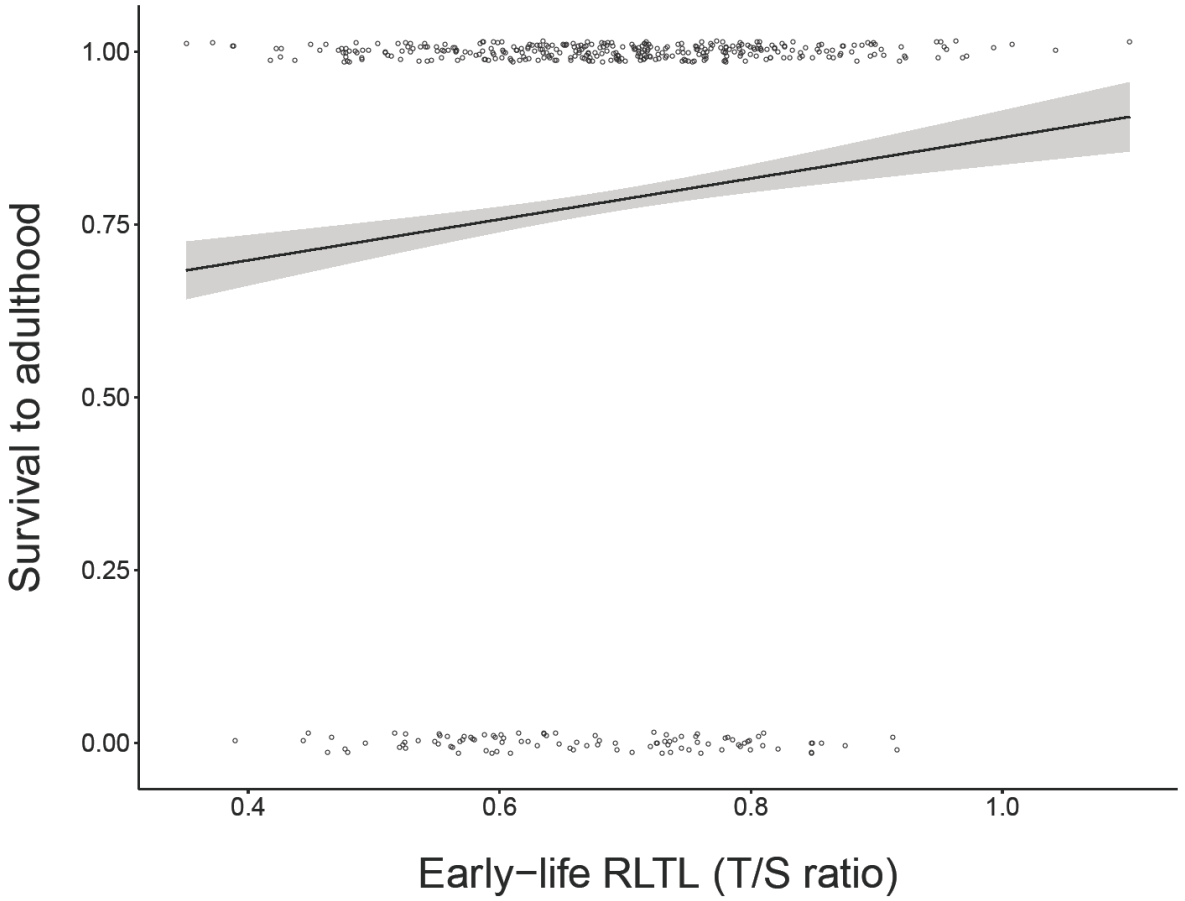

Figure 3: Survival to adulthood (>1 year old) predicted by early-life (<1 year old) relative leukocyte telomere length (RLTL). The regression line from a binomial GLMM is shown, with associated 95% confidence interval as a shaded area, and raw jittered data as open circles (*n* = 435).

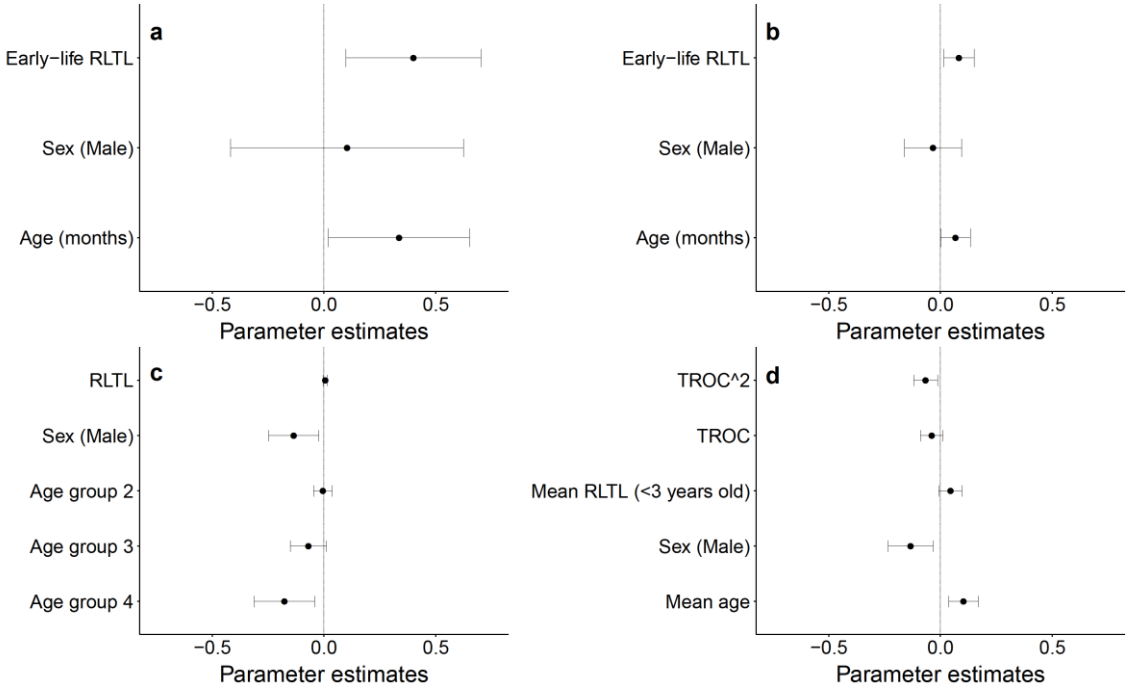

Figure 4: Estimates and 95% confidence intervals for explanatory variables in a) Survival to adulthood – Early-life RLTL (relative leukocyte telomere length), b) Lifespan – Early-life RLTL, c) Residual lifespan – RLTL, d) Lifespan – Telomere rate of change (TROC) mixed models. For cohort effects see figure S3.

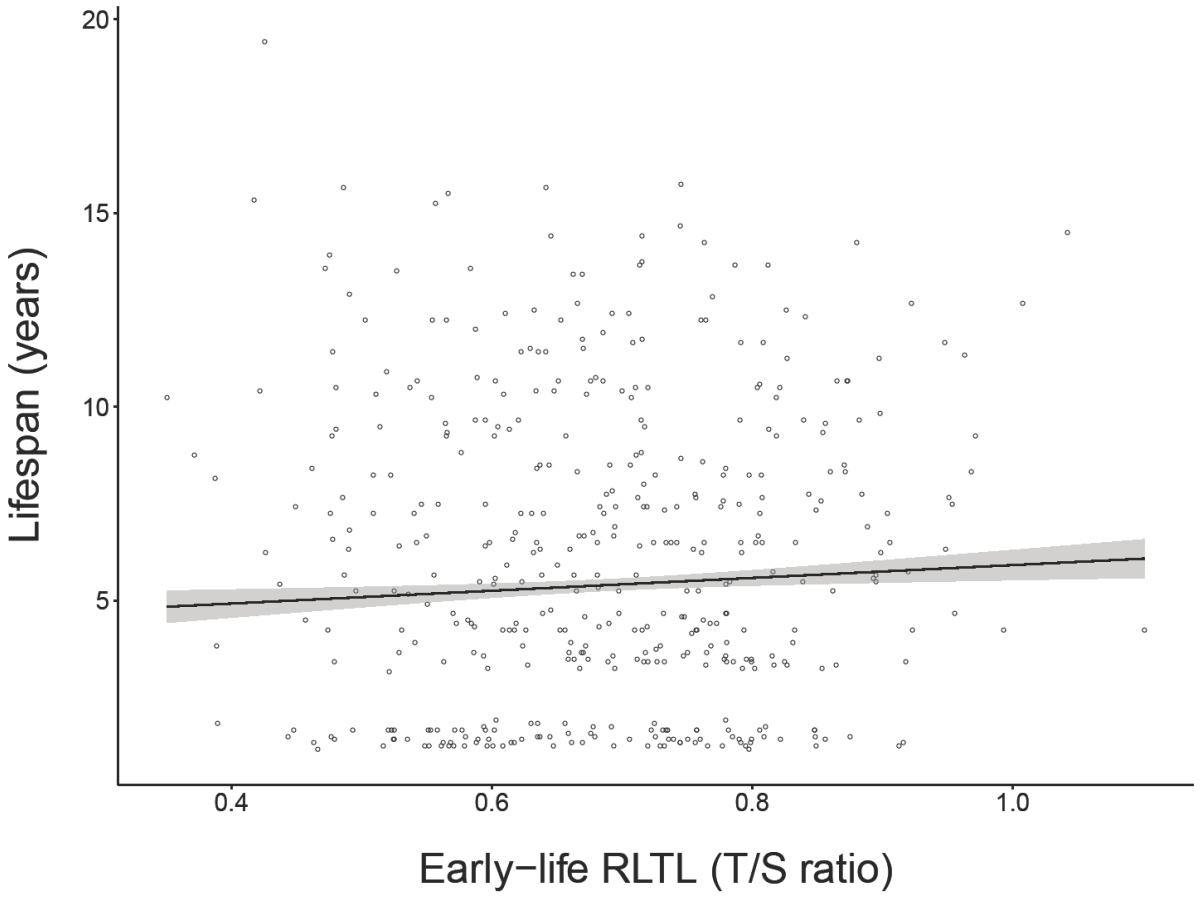

Figure 5: Early-life (<1 year old) relative leukocyte telomere length (RLTL) predicts lifespan. Raw data (*n* = 435) are shown as open circles, the regression from the GLMM as a black line, and the 95% confidence interval as the shaded area.

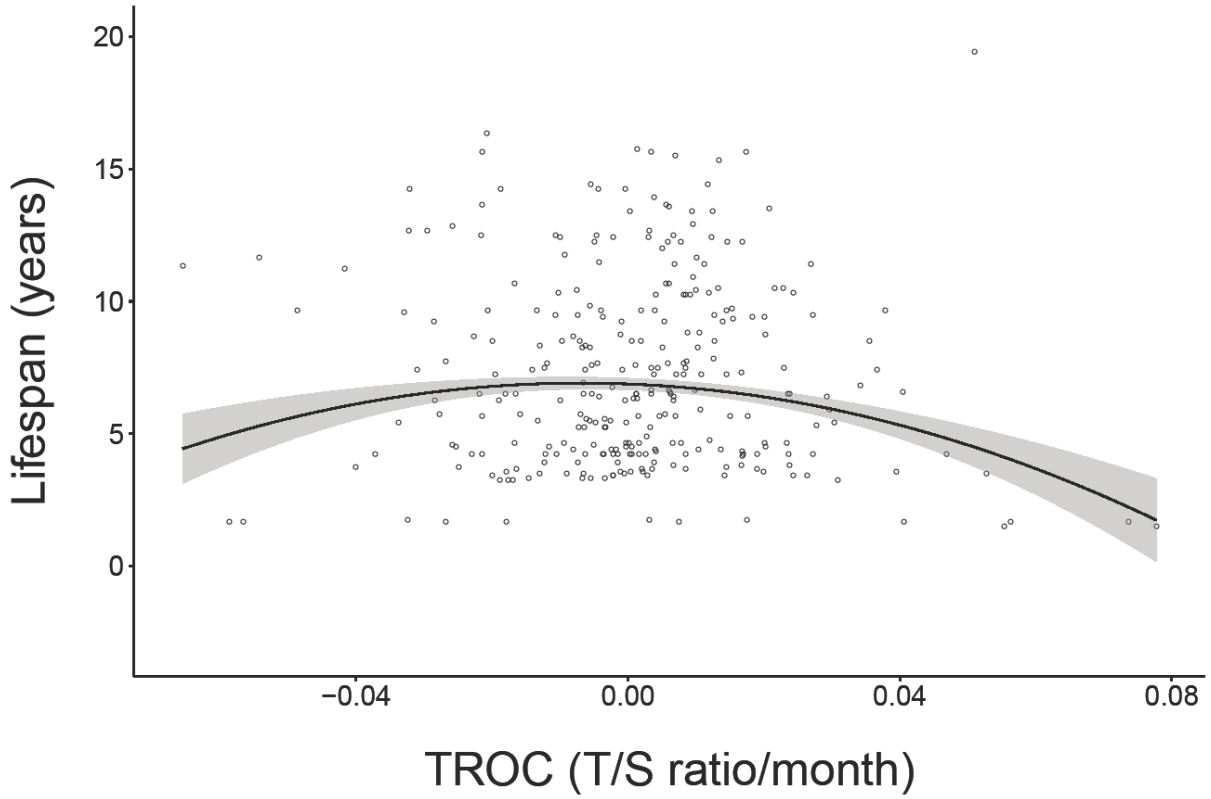

Figure 6: Quadratic relationship between lifespan and telomere rate of change (TROC). Raw data (*n* = 291) are shown with a regression from the GLMM, and the 95% confidence intervals as the shaded area.

Supplementary information

Early-life telomere length predicts survival to adulthood and lifespan in a wild mammal

Sil H.J. van Lieshout, Amanda Bretman, Chris Newman, Christina D. Buesching, David W. Macdonald & Hannah L. Dugdale

This supplementary materials document includes a detailed description of the monochrome multiplex quantitative PCR method and supplementary results, tables and figures.

Supplementary methods

Telomere length estimation through quantitative PCR

Relative leukocyte telomere length (RLTL) was assessed using monochrome multiplex quantitative PCR (MMQPCR), following (Cawthon 2009), to determine the relative amount of telomeric sequence to that of a constant copy number of a control gene (T/S ratio). Running the qPCR analyses in multiplex should, in principle, reduce measurement error as both the telomere sequence and control gene are estimated in the same well, which excludes potential pipetting errors or well effects. Melt-curve analysis (95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec) indicated insufficient differentiation in melt temperatures between the initial primer sets of telomeres and control gene. We therefore added a GC-clamp to the control gene primers to raise the melting temperature. For telomere reactions, 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') were used (Cawthon 2009). The control gene, used previously in badger telomere studies (Beirne *et al.* 2014), was the inter-photoreceptor retinoid-binding protein (IRBP). For IRBP-reactions the GC-clamped primers IRBP-F (5'-CGG-CGG-CGG-GCG-GCG-CGG-GCT-GGG-CGG-GCC-ACA-TTT-CTG-GTA-TCC-CCT-3') and IRBP-R (5'-GCC-CGG-CCC-GCC-GCG-CCC-GTC-CCG-CCG-GGG-CGG-TCG-TAG-ATG-GTA-TC-3') were used. Subsequent melt-curve analyses confirmed differential melt-curves and lack of primer-dimer formation. The protocol was optimised (i.e. the number of PCR cycles) to run both sets of primers in multiplex qPCR reactions.

Semi-skirted 96-well polypropylene qPCR plates were loaded manually with initial reaction volumes of 20 µl. Each well contained 10 µl of SYBR® Select Master Mix (Applied Biosystems, Warrington, UK) , 4.9 µl of nuclease free water, 0.9 µM of both the forward and reverse primers (900 nM) and 1.5 µl of 20 ng/µl DNA sample (which was replaced with 1.5 µl of nuclease free water in controls) and sealed with PCR-plate film adhesive. The cycling conditions in the Quantstudio 12K flex real-time PCR system (Applied Biosystems, Warrington, UK) were: an initial step of 50°C for 2 min and 95°C for 2 min, followed by 2 cycles at 94°C for 15 sec and 49°C for 15 sec, then 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 10 sec and extension at 74°C for 15 sec, 84°C for 10 sec and 86°C for 15 sec.

Samples were randomly allocated to plates and run in duplicate. In addition to our samples, we included a negative control and a serially diluted (4x from 80 to 0.3125 ng/µl) 'golden sample', with the 20 ng/µl sample representing the calibrator sample. No template control reactions amplified for the IRBP and telomere reactions as controls had very high Cq-values (35.94 ± 1.77 SD for telomeres and 36.69 ± 1.56 SD for IRBP), which were far greater than the highest Cq for our samples (27.91). This strongly indicated that any amplification was due to primer dimer formation. We checked that the Cq-values declined in a log-linear fashion (r^2>0.99) before analysing a plate, to ensure there was no effect of initial sample DNA concentration on the estimated Cq-values and the actual data fitted the standard curve well. Amplicon lengths and telomere sequences were confirmed using the Agilent TapeStation 4200 and 3730 DNA Analyzer (Applied Biosystems) with the Big Dye 3.1 cycle sequencing kit.

LinRegPCR 2017.1 (Ruijter *et al.* 2009) was used to correct for baseline fluorescence, to determine the window of linearity for each amplicon (i.e. separate windows for IRBP and telomere

reactions) and to calculate amplification efficiencies for each well. Subsequently, Cq-values for each sample were calculated in R 3.3.1 (R Development Core Team 2018). Across plates (n = 34), fluorescence thresholds (Nq) were set to a constant value within the window of linearity for the amplification curves: 0.432 for IRBP and 0.694 for telomeres. Mean amplification efficiency across wells for each amplicon group per plate, excluding outliers (outside the 5th and 95th percentiles), were used as our estimates of reaction efficiency (as recommended by Ruijter *et al.* 2009).

Further quality control was applied, where samples were excluded from further analyses if the standard deviation across their duplicate Cq values for either amplicon group was greater than 5% of the mean Cq for that sample (*n* = 25). We also excluded any sample if the standard deviation across the duplicate well-specific efficiencies for either amplicon was greater than 5% of the overall mean efficiency for that amplicon group (*n* = 44). Lastly, samples with a Cq-value >28 for telomere, or >29 for IRBP, were excluded from the analysis (*n* = 24), assuming that these were failed reactions. In order to determine failed reactions for control samples, we applied a similar rule where samples with a standard deviation of the duplicate T/S ratios >8% of the mean T/S ratio for that sample were excluded, as at least one of the duplicate samples was assumed to have failed (*n* = 19; <12% of samples).

Reaction efficiencies differed between our IRBP and telomere reactions (mean efficiencies across all samples on all plates run: IRBP = 1.793 ± 0.004 SE; Telomere: 1.909 ± 0.004 SE). Assuming constant amplification efficiencies across plates can bias qPCR results when these actually differ, we therefore calculated relative leukocyte telomere length (RLTL) using a method (Pfaffl 2001) that does not assume consistent efficiencies across plates (Fairlie *et al.* 2016):

$$RLTL = \frac{(E_{tel}\text{^}(Cq_{tel(calibrator)} - Cq_{tel(sample)}))}{(E_{IRBP}\text{^}(Cq_{IRBP(calibrator)} - Cq_{IRBP(sample)}))}$$

In this equation, E_{tel} and E_{IRBP} represent the mean well efficiencies for each of the amplicons, calculated in LinRegPCR, $Cq_{tel(calibrator)}$ and $Cq_{IRBP(calibrator)}$ are the mean Cq-values for the calibrators (20 ng/µl) for each amplicon and $Cq_{tel(sample)}$ and $Cq_{IRBP(sample)}$ are the mean Cq-values for both amplicons in each sample.

Only 69 of 1324 samples did not pass the initial quality control and these samples were repeated. 17 repeated samples passed the quality control, meaning that 52 samples (<4%) were excluded. Additionally, as 24 samples had a Cq-value >28 for TL or Cq-value >29 for IRBP, which we considered to be failed reactions, these were excluded from the analyses. This resulted in a total of 1248 RLTL measurements from 612 individuals (308 males and 304 females), with 163 individuals having 1 sample (early-life samples to reduce bias from viability selection), 408 individuals with 2 samples, 5 individuals with 5 samples, 17 individuals with 6 samples, 12 individuals with 7 samples, 5 individuals with 8 samples and 2 individuals with 9 samples.

Inter-plate repeatability (intraclass correlation coefficient), calculated with rptR 0.9.2 (Stoffel *et al.* 2017), was 0.82 (95% CI = 0.77 – 0.87). Intra-plate repeatability was 0.83 (95% CI = 0.77 – 0.90) and 0.86 (95% CI = 0.79 – 0.92) for IRBP and telomere Cq values, respectively.

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Results - summary of analyses excluding individuals aged based on tooth wear

The following summary results represent the exact same analyses, but without the 67 individuals whose age was determined based on tooth wear

Individual repeatability was 0.025 (95% CI = 0.023 – 0.055), (marginal R^2 = 0.014; X^2 = 2.47, P = 0.116).

Telomere elongation was observed in in 56.4% of within-individual changes. Variance among within-individual samples was higher than variance among technical replicates (Levene's test, $F_{1,1121}$ = 1086.5, P < 0.001), for both samples that increased (Wilcoxon test, P < 0.001) and that decreased in telomere length (Wilcoxon test, P < 0.001). Residual variance ($\sigma_{\varepsilon}^{-2}$) was smaller (0.021) than the overall change ($\sigma_{\varepsilon}^{'2}$) in RLTL (0.450; $F_{17,16}$ = 21.88, P < 0.001).

Early-life RLTL predicted survival to adulthood (β = 0.409, 95% CI = 0.110 – 0.708), while early-life RLTL also showed a positive relationship with lifespan (β = 0.085, 95% CI = 0.014 – 0.156). Residual lifespan was not predicted by the respective RLTL (β = 0.007, 95% CI = -0.004 – 0.017). Lifespan showed a quadratic relationship with TROC (β = -0.077, 95% CI = -0.138 – -0.015) and individuals with longer mean early-life RLTL did not show a more rapid shortening of RLTL (β = 0.002, 95% CI = -0.001 – 0.004).

The addition of individuals aged using tooth wear did therefore not alter any results or conclusions.

Supplementary tables and figures

Table S1: Comparison of models describing the relationship between relative leukocyte telomere length and age, with a variety of age functions (0 = no age function, 1 = linear age function, 2 = log age function, 3 linear and quadratic age function, F = factorial age function, T1 = single threshold, T2 = double threshold, T3 = triple threshold) and cohort, including its interaction with age (cohort * age), controlling for plate, year, individual ID and sample storage time. Models were ordered and numbered by AIC, and the difference from the top model (lowest AIC) is stated in the column termed 'Delta AIC'.

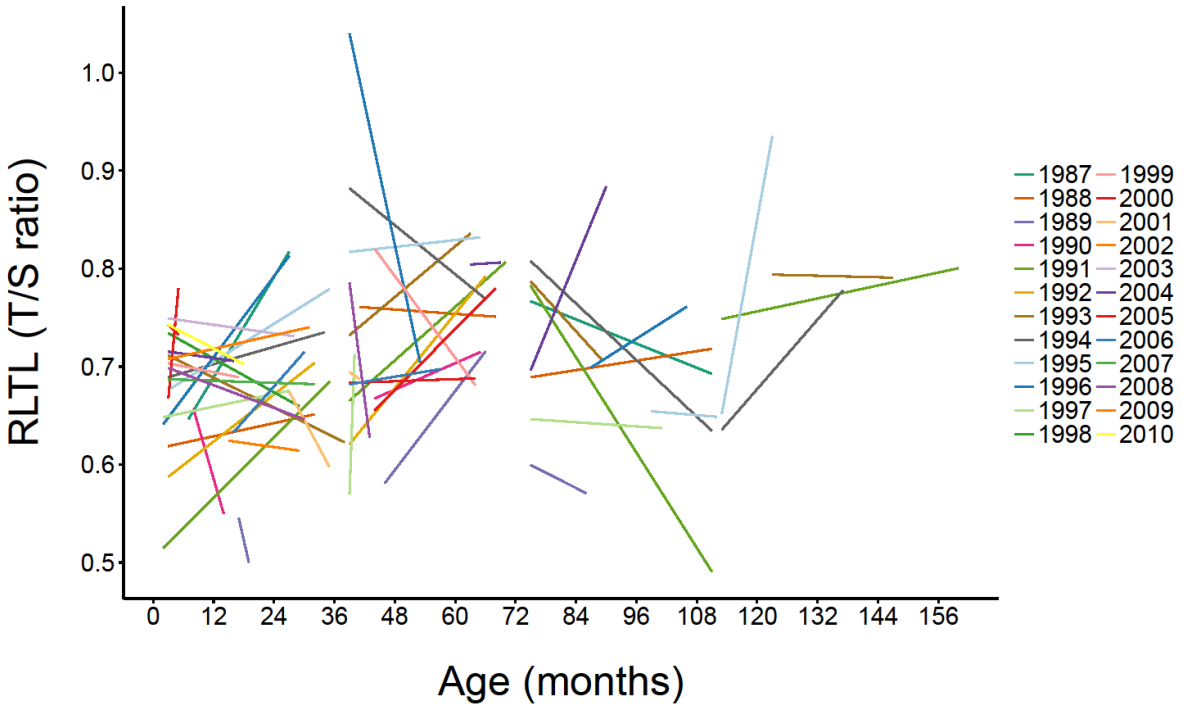
Model No.	Function	Threshold 1	Threshold 2	Threshold 3	Cohort	Cohort*Age	Degrees of freedom	AIC	Delta AIC
1	**T3**	**38**	**74**	**112**	**Yes**	**No**	**32**	**-1706.101**	
2	**T2**	**38**	**112**		**Yes**	**No**	**31**	**-1704.703**	1.398
3	**T1**	**38**			**Yes**	**No**	**30**	**-1704.005**	2.096
4	**T2**	**38**	**74**		**Yes**	**No**	**31**	**-1703.474**	2.627
5	1				Yes	No	30	-1694.672	11.429
6	3				Yes	No	31	-1694.060	12.041
7	2				Yes	No	30	-1693.415	12.686
8	T1	112			Yes	No	30	-1689.461	16.64
9	T2	74	112		Yes	No	31	-1688.535	17.566
10	0				Yes	No	29	-1688.509	17.592
11	T3	38	74	112	No	No	9	-1686.943	19.158
12	T1	74			Yes	No	30	-1686.759	19.342
13	T2	38	112		No	No	8	-1685.984	20.117
14	T1	38			No	No	7	-1685.301	20.8
15	T1	112			Yes	Yes	37	-1684.652	21.449
16	T2	38	74		No	No	8	-1684.150	21.951
17	T1	38			Yes	Yes	49	-1682.860	23.241
18	T1	112			No	No	7	-1682.722	23.379
19	1				No	No	7	-1681.442	24.659
20	T2	74	112		No	No	8	-1681.161	24.94
21	0				No	No	6	-1680.294	25.807
22	T2	38	112		Yes	Yes	57	-1680.010	26.091
23	2				No	No	7	-1679.906	26.195
24	3				No	No	8	-1679.493	26.608
25	T1	74			No	No	7	-1678.354	27.747
26	T2	74	112		Yes	Yes	48	-1672.890	33.211
27	T1	74			Yes	Yes	42	-1670.427	35.674
28	2				Yes	Yes	53	-1668.265	37.836
29	T3	38	74	112	Yes	Yes	68	-1668.079	38.022
30	T2	38	74		Yes	Yes	62	-1667.997	38.104
31	1				Yes	Yes	53	-1661.564	44.537
32	3				Yes	Yes	77	-1635.616	70.485
33	F				Yes	No	117	-1619.689	86.412
34	F				No	No	94	-1608.165	97.936
35	F				Yes	Yes	379	-1221.852	484.249

Table S2: Model averaged parameters of models. ∑ = relative variable importance, β = direction and magnitude of effect, S.E. = standard error, CI = confidence interval; with reference terms in brackets = reference level for factors (with additional levels below), for cohort effect estimates see Figure S3.

Parameter (reference level)	∑	β	S.E.	95% CI
Survival to adulthood[1]				
Early-life RLTL	0.96	0.399	0.154	0.098 to 0.702
Sex (male)	0.25	0.103	0.265	-0.417 to 0.624
Cohort	0.93			
Linear age	0.80	0.336	0.161	0.020 to 0.652
Lifespan (Early-life RLTL)[2]				
Early-life RLTL	0.84	0.083	0.035	0.014 to 0.151
Sex (male)	0.27	-0.033	0.065	-0.160 to 0.093
Cohort	1.00			
Linear age	0.70	0.067	0.034	0.001 to 0.134
Residual lifespan[3]				
RLTL	0.39	0.005	0.005	-0.004 to 0.015
Sex (male)	0.86	-0.135	0.057	-0.247 to -0.023
Cohort	1.00			
Age (>38, <= 74 months)	0.93	-0.005	0.020	-0.046 to 0.036
(>74, <= 112 months)		-0.070	0.041	-0.151 to 0.011
(> 112 months)		-0.177	0.069	-0.321 to -0.042
Lifespan (TROC)[4]				
TROC	0.50	-0.039	0.026	-0.090 to 0.011
TROC^2	0.88	-0.067	0.027	-0.120 to -0.013
Mean RLTL (≤3 yrs old)	0.57	0.045	0.026	-0.007 to 0.096

Sex (male)	0.91	-0.134	0.051	-0.234 to -0.032
Cohort	0.56			
Mean age	0.96	0.103	0.034	0.036 to 0.169
TROC – Initial RLTL[5]				
Mean RLTL (≤3 yrs old)		0.002	0.001	-0.001 to 0.004
Sex (male)		0.001	0.002	-0.005 to 0.004
Cohort				
Mean age		-0.002	0.002	-0.005 to 0.001

Model averaged random effect estimates (variance ± SD): [1]Storage time (0.038 ± 0.052), Plate (0.003 ± 0.044), Natal social group (0.277 ± 0.525); [2]Observation (0.389 ± 0.623), Storage time (0.000 ± 0.000), Plate (4.310^{e-11} ± 1.847^{e-06}), Natal social group (0.072 ± 0.269); [3]Individual ID (0.446 ± 0.667), Storage time (1.274^{e-04} ± 0.011), Plate (0.000 ± 0.000), Natal social group (0.080 ± 0.283), Social group (0.005 ± 0.068), Year (1.132 ± 1.064); [4]Observation (0.129 ± 0.359), Storage time (1.363^{e-09} ± 1.886^{e-05}), Plate (0.011 ± 0.103), Natal social group (0.023 ± 0.124), Social group (0.012 ± 0.064), Year (0.026 ± 0.108); [5]consisted of one model: Storage time (4.056^{e-07} ± 6.368^{e-04}), Plate (2.421^{e-06} ± 1.556^{e-03}), Natal social group (1.121^{e-05} ± 3.349^{e-03}), Year (4.282^{e-05} ± 6.544^{e-03}).



876

877 **Figure S1**: Variation in relative leukocyte telomere length (RLTL) among 24 cohorts across four age
878 groups defined by threshold modelling. Fitted regression lines are shown. Raw data points and 95%
879 confidence intervals are not shown for clarity.

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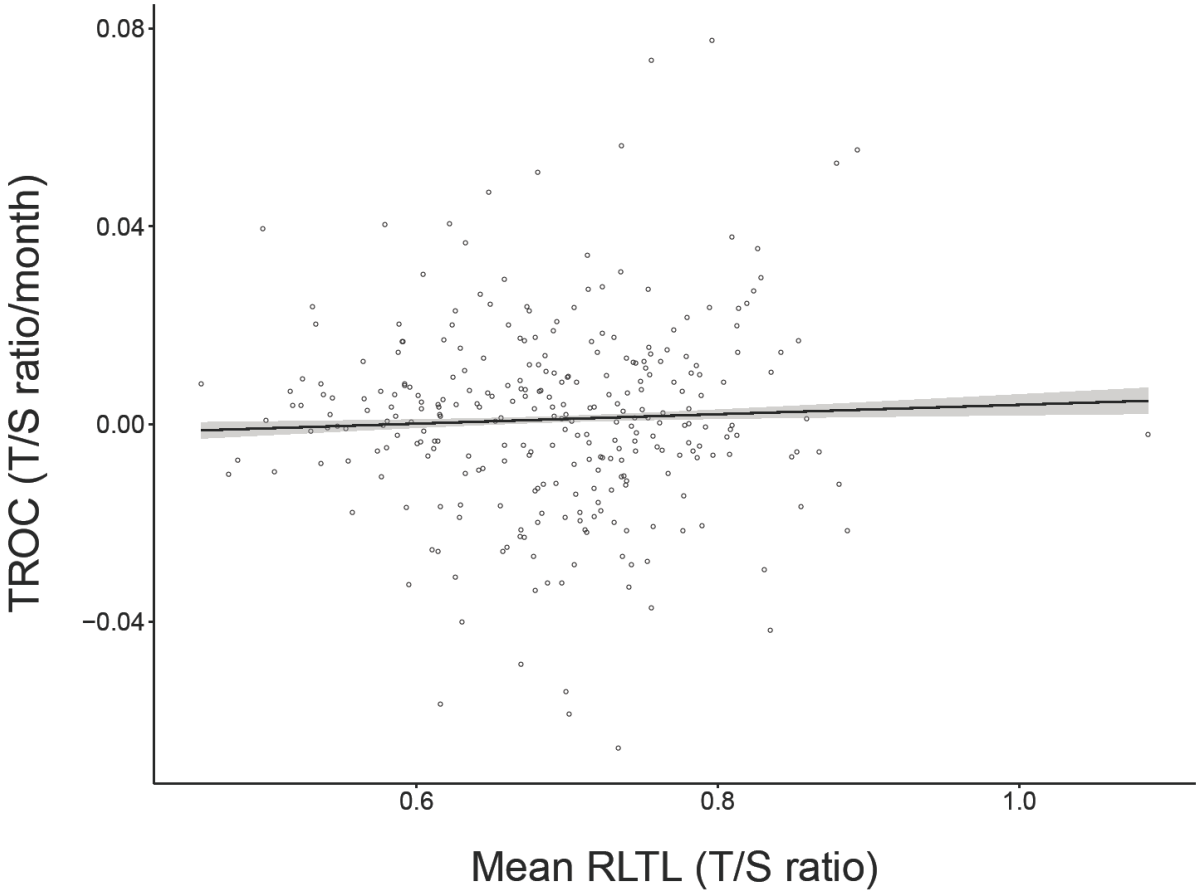
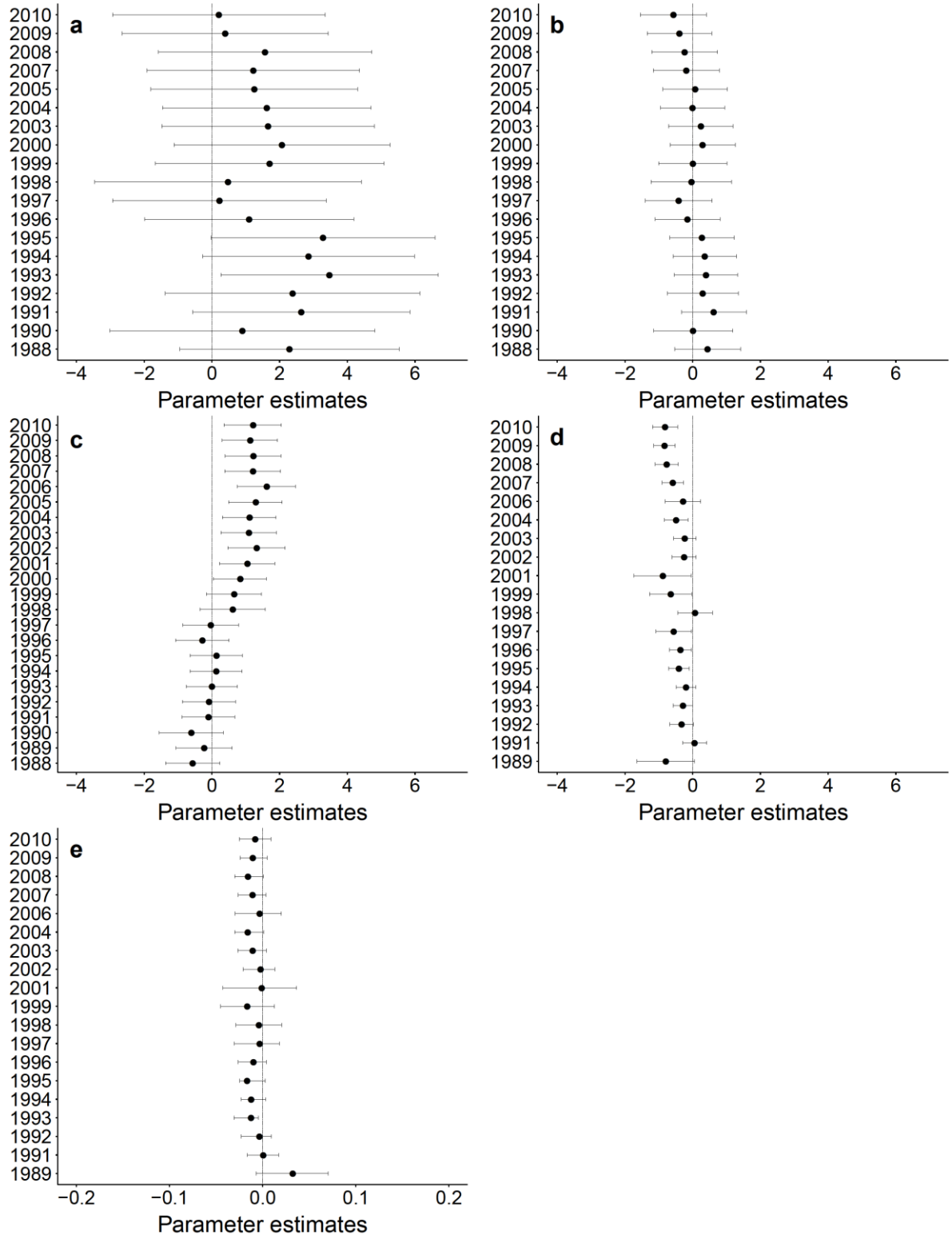


Figure S2: Relationship between early-life relative leukocyte telomere length (RLTL) and early-life telomere rate of change (TROC). Raw data (*n* = 291) are shown as open circles, the regression from the GLMM as a black line, and the 95% confidence interval as the shaded area.



886

887 **Figure S3:** Estimates and 95% confidence intervals for cohort effects in models of: a) Survival to
888 adulthood – Early-life RLTL (relative leukocyte telomere length), b) Lifespan – Early-life RLTL, c) Residual
889 lifespan – RLTL, d) Lifespan – Telomere rate of change (TROC) and e) TROC – mean RLTL (<3 years old)
890 mixed models. Scales differ in plot e).

891