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Estimation of environmental, genetic and parental age at conception effects on telomere length in a wild mammal

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Abstract

Understanding individual variation in fitness-related traits requires separating the environmental and genetic determinants. Telomeres are protective caps at the ends of chromosomes that are thought to be a biomarker of senescence as their length predicts mortality risk and reflect the physiological consequences of environmental conditions. The relative contribution of genetic and environmental factors to individual variation in telomere length is however unclear, yet important for understanding its evolutionary dynamics. In particular, the evidence for transgenerational effects, in terms of parental age at conception, on telomere length is mixed. Here, we investigate the heritability of telomere length, using the 'animal model', and parental age at conception effects on offspring telomere length in a wild population of European badgers (*Meles meles*). While we found no

heritability of telomere length and low evolvability (<0.001), our power to detect heritability was low and a repeatability of 2% across individual lifetimes provides a low upper limit to ordinary narrow-sense heritability. However, year (25%) and cohort (3%) explained greater proportions of the phenotypic variance in telomere length. There was no support for cross-sectional or within-individual parental age at conception effects on offspring telomere length. Our results indicate a lack of transgenerational effects through parental age at conception and a low potential for evolutionary change in telomere length in this population. Instead, we provide evidence that individual variation in telomere length is largely driven by environmental variation in this wild mammal.

Keywords: Telomere length, heritability, parental age at conception, senescence, wild mammal

1. Introduction

The extrinsic environment can have individual-specific effects on physiology, which are key to variation in fitness (Lindström, 1999), life-history strategies (Metcalfe & Monaghan, 2001) and senescence patterns (Nussey, Kruuk, Morris, & Clutton-Brock, 2007). However, in wild populations it is challenging to quantify how the extrinsic environment affects physiology. Consequently, biomarkers reflecting how such physiological costs are related to fitness are required. The forces of natural selection acting on the heritability of such a biomarker (the proportion of phenotypic variance explained by additive genetic variance), can describe its evolutionary potential (Charmantier, Brommer, & Nussey, 2014; Lynch & Walsh, 1998). It is therefore important to separate environmental and genetic components that contribute to individual variation in fitness-related traits in order to understand the evolution of such traits (Charmantier et al., 2014; Wilson, Charmantier, & Hadfield, 2008).

Telomeres are a biomarker of senescence in some species (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Monaghan & Haussmann, 2006), and understanding the heritability and evolvability of telomere length may provide insight into the evolution of senescence (Dugdale &

Richardson, 2018). In addition, telomeres can quantify the physiological costs incurred by environmental conditions (Monaghan, 2014). Telomeres are repetitive non-coding sequences (5'-TTAGGG-3') at the ends of eukaryotic chromosomes that, along with shelterin proteins, maintain genomic integrity and prevent end-to-end fusion of linear chromosomes (Blackburn, 1991; de Lange, 2005). Due to the end-replication problem, telomeres shorten with each cell division (Olovnikov, 1973). Telomere shortening can, however, be accelerated by adverse environmental conditions (e.g. Boonekamp, Mulder, Salomons, Dijkstra, & Verhulst, 2014; Nettle et al., 2015) and metabolically demanding activities (Epel et al., 2004; Heidinger et al., 2012). *In vitro* evidence shows that oxidative damage contributes to telomere shortening (von Zglinicki, 2002), but there is no evidence for such effects *in vivo* (Boonekamp, Bauch, Mulder, & Verhulst, 2017; Reichert & Stier, 2017). Telomeres can also be restored by telomerase, although this enzyme is transcriptionally repressed in adult somatic tissue in many large-bodied endothermic vertebrates (Blackburn et al., 1989; Gomes, Shay, & Wright, 2010). However, alternative telomere lengthening pathways exist (Cesare & Reddel, 2010; Mendez-Bermudez et al., 2012). Critically, short telomeres can result in replicative senescence, where accumulation of senescent cells can impair tissue functioning (Armanios & Blackburn, 2012; Campisi, 2005), and may lead to organismal senescence (Young, 2018).

1.1 Heritability of telomere length

Individual variation in telomere length occurs in wild populations (Fairlie et al., 2016; Spurgin et al., 2017; van Lieshout et al., 2019) which is linked to individual life-histories (Wilbourn et al., 2018). Understanding the degree to which individual variation in telomere length is due to genetic and environmental effects, in addition to the strength of natural selection acting on telomere length, allows estimation of the potential for evolutionary change (Charmantier et al., 2014; Lynch & Walsh, 1998). Heritability of telomere length has been estimated in over seven wild species and in >26 studies in humans (see Table 1 in Dugdale & Richardson, 2018). These studies primarily used parent–offspring regressions to determine the heritability of telomere length, with estimates ranging from 0 to 1. The

majority, however, of these heritability estimates were relatively high, which is unexpected given that heritabilities of traits closely related to fitness are often low (Mousseau & Roff, 1987; Postma, 2014; Price & Schluter, 1991). However, parents and offspring often live in similar environments, and parent–offspring regressions are frequently confounded by these 'shared environment' effects, which can inflate heritability estimates (Kruuk, 2004).

The 'animal model' provides a statistical approach that can overcome the drawbacks of parent–offspring regressions because it allows partitioning of variance components into additive genetic and shared environment sources (Kruuk & Hadfield, 2007; Wilson et al., 2010). Because narrow-sense heritability is the proportion of phenotypic variation due to additive genetic variance, any changes to the amount of environmental variation will impact heritability estimates, even if the additive genetic variance does not itself change (Dugdale & Richardson, 2018; Kruuk & Hadfield, 2007). Environmental effects (e.g. Boonekamp et al., 2014; Nettle et al., 2015) therefore need to be accounted for to derive accurate heritability estimates (Dugdale & Richardson, 2018). The 'animal model' is a mixed-effects model that uses either the expected proportion of the genome that individuals share by descent (from a pedigree) or by state (from genomic data) to partition phenotypic variance into environmental and genetic components (Wilson et al., 2010).

The three studies applying an animal model approach in wild populations of non-human vertebrates found no heritability of telomere length in white-throated dippers (Cinclus cinclus; 0.007 ± 0.013 SE; Becker et al., 2015), low heritability in *Myotis* bats (Myotis myotis; from 0.011, 95% CI = 0.000–0.042 to 0.060, 95% CI = 0.023–0.106 depending on prior specification; Foley et al., 2020), but high heritability in great reed warblers (Acrocephalus arundinaceus; 0.480 ± 0.120 SE; Asghar, Bensch, Tarka, Hansson, & Hasselquist, 2015). However, although these were pioneering studies, some of the sample sizes were relatively low for quantitative genetic analyses and the power to detect heritability was not stated. Additionally, two of these studies did not have repeated measures to estimate permanent environment effects, which may inflate additive genetic effects (Kruuk & Hadfield, 2007). More studies in wild populations, and from a wider range of taxa, with larger sample sizes and

repeated measures, are required to distentangle the genetic and environmental contributions to variation in telomere length.

The influence of environmental conditions on variation in telomere length is not only important to account for statistically, but informs about which environmental factors shape individual telomere length. Previous studies have shown that cohort (i.e. birth year; Fairlie et al., 2016; Hall et al., 2004; Watson, Bolton, & Monaghan, 2015), year (Mizutani, Tomita, Niizuma, & Yoda, 2013; Wilbourn et al., 2017), social group (Boonekamp et al., 2014; Cram, Monaghan, Gillespie, & Clutton-Brock, 2017; Nettle et al., 2015) and parental effects (Asghar et al., 2015; Cram et al., 2017) affect individual telomere length. Understanding the relative contribution of these different sources of environmental variation on telomere length sheds light on its evolution. Additionally, for insight into the evolutionary potential of telomere length, evolvability (a standardised measure of additive genetic variance) facilitates comparison of the evolutionary potential of the same trait in different populations and species (Houle, 1992).

1.2 Parental age at conception effects

In addition to these environmental and additive genetic effects, offspring telomere length may also be influenced by negative paternal age at conception (PAC) effects due to sperm telomeres shortening with age (de Frutos et al., 2016), or positive PAC effects according to two mutually non-exclusive hypotheses. First, to compensate for telomere loss due to sperm production and progressive cell replication, telomerase activity in germ stem cells is high. Telomerase expression might, beyond restoring telomere length, overcompensate and result in elongation of telomeres in germ stem cells (Aviv & Susser, 2013; Kimura et al., 2008). Second, stem cells with longer telomeres are better able to withstand repeated cell replication and therefore may become predominant in the stem cell pool with age due to the selective loss of germ stem cells with shorter telomeres (Hjelmborg et al., 2015; Kimura et al., 2008).

In humans, there is cross-sectional evidence that older men produce sperm with longer telomeres (r = 0.127 – 0.160; Aston et al., 2012; de Meyer et al., 2007; Kimura et al., 2008; Nordfjall, Svenson, Norrback, Adolfsson, & Roos, 2010). The evidence for a positive cross-sectional PAC effect is even stronger in captive chimpanzees (*Pan troglodytes*; *r* = 0.378) compared to humans (Eisenberg, Tackney, Cawthon, Cloutier, & Hawkes, 2017). An explanation for this stronger effect is that chimpanzees have relatively larger testes and higher rates of sperm production than humans, due to their more promiscuous mating system (Birkhead & Møller, 1998). Stronger sperm competition could therefore result in the PAC effect, because stronger postcopulatory competition should select for high quality sperm to be produced at a fast rate (Eisenberg et al., 2017). We would therefore expect that species with high levels of sperm competition and high rates of sperm production, such as in polygynandrous species, should show the strongest PAC effect.

PAC effects are often confounded with maternal age at conception (MAC), as these are typically highly correlated in human populations (Table 1 in Froy et al., 2017). The presence of MAC effects in humans is generally considered to be due to the correlation with PAC instead of a true independent biological effect (de Meyer et al., 2007; Kimura et al., 2008), because oocytes are produced prenatally, while sperm is produced throughout life (Eisenberg & Kuzawa, 2018). However, MAC effects may occur if oocyte quality differs such that there is selection for better quality oocytes, with longer telomeres, to be used earlier in life (Duran, Simsek-Duran, Oehninger, Jones, & Castora, 2011; Monaghan, Maklakov, & Metcalfe, 2020).

Parental age effects on offspring fitness may also be sex-specific (Bouwhuis, Vedder, & Becker, 2015). For example, male house sparrows (*Passer domesticus*) with older fathers and females with older mothers had lower lifetime reproductive success, with sex-specific telomere shortening hypothesised to be a potential mechanism (Schroeder, Nakagawa, Rees, Mannarelli, & Burke, 2015). However, there was no evidence of sex-specific offspring telomere length underlying sex-specific parental age effects on offspring reproductive success in common terns (Sterna hirundo; Bouwhuis, Verhulst, Bauch, & Vedder, 2018) and sex-specific telomere lengths are rare in birds (Barrett &

156 Richardson, 2011). Additionally, PAC effects on offspring lifespan and telomere length in captive zebra
157 finch (*Taeniopygia guttata*) were not offspring-sex-specific: offspring from older parents had reduced
158 lifespan, and embryos from the same mother with older versus younger fathers had shorter telomere
159 lengths (Noguera, Metcalfe, & Monaghan, 2018). Parental age at conception effects may therefore
160 differ according to offspring sex, but this is rarely tested in wild populations.

161 Studies in wild populations have provided mixed evidence for PAC and MAC effects. Studies
162 from different taxa, with a variety of mating systems, have shown a negative PAC effect (Bouwhuis et
163 al., 2018; Criscuolo, Zahn, & Bize, 2017; Olsson et al., 2011), including a longitudinal (Bauch,
164 Boonekamp, Korsten, Mulder, & Verhulst, 2019) and an experimental study (Noguera et al., 2018).
165 However, other studies have reported no PAC or MAC effect on offspring telomere length (Belmaker,
166 Hallinger, Glynn, Winkler, & Haussmann, 2019; Froy et al., 2017; Heidinger et al., 2016; McLennan et
167 al., 2018), a positive MAC effect (Asghar et al., 2015) or a positive mean parental age effect (Dupont
168 et al., 2018). The variation in PAC and MAC effects on offspring telomere length among species
169 requires more studies to disentangle potential causes and mechanisms underlying such variation in
170 transgenerational effects.

171

172 *1.3 Testing heritability and parental age effects in European badgers*

173 Here, we investigate PAC and MAC effects and the heritability of telomere length in polygynandrous
174 European badgers (*Meles meles*; henceforth 'badgers'). Individual variation in badger telomere length
175 in early-life (<1 year old), but not adult life, is predictive of survival probability (van Lieshout et al.,
176 2019). However, a low heritability is expected, as within-individual repeatability in telomere length is
177 very low (0.022, 95% CI = 0.001 – 0.103; van Lieshout et al., 2019). While this sets the upper limit for
178 ordinary narrow-sense heritability (Bijma, 2011), understanding the relative importance of
179 environmental (i.e. cohort, year, social group, maternal and paternal effects) and additive genetic
180 variance components is important to understand the evolution of telomere length. Badgers respond
181 to year-specific weather variation which affects their behaviour, physiology and fitness (Bilham et al.,

2018; Macdonald, Newman, Buesching, & Nouvellet, 2010; Noonan et al., 2014; Nouvellet, Newman, Buesching, & Macdonald, 2013) and because they are group-living, they may be impacted by social group attributes (Beirne, Delahay, & Young, 2015; Woodroffe & Macdonald, 2000). Cubs are born synchronously in February, which is followed by a post-partum mating peak, after which matings can occur throughout the year (Macdonald, Newman, & Buesching, 2015). Badgers are highly promiscuous, which may promote sperm competition (Dugdale, Griffiths, & Macdonald, 2011). However, male badgers' testes ascend in autumn/winter (Woodroffe & Macdonald, 1995), leading to reduced sperm production rates (Sugianto, Newman, Macdonald, & Buesching, 2019), and with the lack of continuity in sperm production this may reduce the potential for transgenerational effects (i.e. PAC/MAC effects) on offspring telomere length (Bouwhuis et al., 2018).

We therefore test for: (i) sex-specific and longitudinal PAC and MAC effects on offspring relative leukocyte telomere length (RLTL), after assessing whether PAC and MAC are correlated; and (ii) the proportion of variance in juvenile RLTL (≤29 months old) and RLTL across individual lifetimes, that is explained by additive genetic and environmental effects.

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, Newman, Dean, Buesching, & Johnson, 2004). The resident badger population forms an almost closed population (immigration/emigration <3%; Macdonald & Newman, 2002). Badgers live in social groups with a mean of 11.3 individuals (range = 2 – 29; da Silva, Macdonald, & Evans, 1994) and a mean number of 19 social groups (95% CI = 17 – 21; range = 14 – 26; Dugdale, Macdonald, Pope, Johnson, & Burke, 2008) in the population between 1987–2010. Cohort-dependent cub survival probability varied from 0.61 to 0.94 (mean ± SE = 0.67 ± 0.03; Macdonald, Newman, Nouvellet, & Buesching, 2009), whereas mean annual adult survival probability

208 in the population was 0.83 (± 0.01 SE; Macdonald et al., 2009) with a mean lifespan of 3.31 years (±
209 3.51 SD; Bright Ross, J., Pers. Comm.).
210 Trapping sessions were conducted three or four times per year over two weeks in May–June
211 (Spring), August–September (Summer) and November (Autumn), with trapping in January (Winter) in
212 focal years, for two to three consecutive days per social group. Trapped badgers were anaesthetised
213 using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et
214 al., 2005). Badgers were identified by a unique tattoo number on the left inguinal region. Sex, age
215 class, sett (group den system), social group and capture date were recorded for each badger. Badgers
216 were aged by the number of days elapsed since the 14th of February (the averaged date of
217 synchronised parturition) in the respective birth year (Yamaguchi, Dugdale, & Macdonald, 2006).
218 Individuals first caught as adults were aged through tooth wear (on a scale of 1–5), which is commonly
219 used and highly correlated (r^2 = 0.80) with known age in our population (Bright Ross, Newman,
220 Buesching, & Macdonald, 2020; da Silva & Macdonald, 1989; Hancox, 1988; Macdonald et al., 2009)
221 where tooth wear 2 typically indicates a 1-year old adult (van Lieshout et al., 2019). Blood was
222 collected by jugular venipuncture into vacutainers with an EDTA anticoagulant and stored at -20°C
223 immediately. Badgers were released at their setts later on the day of capture, after full recovery from
224 anaesthesia.
225
226 2.2 *Molecular analyses*
227 We extracted genomic DNA from whole blood samples (*n* = 1248 samples; 612 badgers) using the
228 DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, with
229 modifications by conducting a double elution step (2x 75 µl AE buffer) and using 125 µl of
230 anticoagulated blood. We checked DNA integrity by running a random selection of DNA extracts (ca.
231 20%) on agarose gels to ensure high molecular weight, and found no evidence of degredation. DNA
232 concentration of all samples was quantified using the Fluostar Optima fluorometer (BMG Labtech,
233 Ortenberg, Germany) and standardized to 20 ng/µl, after which samples were stored at -20 °C. We

234 used monochrome multiplex quantitative PCR (MMqPCR) analysis to measure RLTL (Cawthon, 2009).
235 This measure is the abundance of telomeric sequence relative to a reference gene, which are both
236 analysed in the same well, and represents the mean telomere length across cells in a sample. Cq-
237 values on the qPCR plates (n = 34) declined in a log-linear fashion ($r^2 > 0.99$). Reaction efficiencies were
238 (mean ± SE) 1.793 ± 0.004 for IRBP and 1.909 ± 0.004 for telomeres. Inter-plate repeatability (intraclass
239 correlation coefficient) calculated from the reference sample was 0.82 for RLTL measurements (95%
240 CI = 0.76–0.87; n = 142 samples; 34 plates), and intra-plate repeatability calculated with duplicates of
241 the same sample on the same plate, while controlling for plate effects, was 0.90 (95%CI = 0.86–0.93;
242 n = 1,248 samples; 34 plates) for IRBP, 0.84 (95%CI = 0.79–0.90; n = 1,248 samples; 34 plates) for
243 telomere Cq-values and 0.87 (95% CI = 0.82–0.91; n = 1,248 samples; 34 plates) for RLTL
244 measurements. A detailed description of the MMqPCR analysis can be found in van Lieshout et al.
245 (2019).
246
247 2.3 *Pedigree*
248 The pedigree was constructed using DNA extracted from blood or guard hair samples, genotyped for
249 35 microsatellite loci (Annavi, Newman, Buesching, et al., 2014; Dugdale, Macdonald, Pope, & Burke,
250 2007), and *MasterBayes* 2.47 (Hadfield, 2010). The pruned pedigree (which excludes non-informative
251 individuals) contained 753 unique individuals, from 7 generations, trapped between 1987 and 2010
252 (Table S1).
253
254 2.4 *Statistical analyses*
255 2.4.1 *PAC and MAC effects*
256 Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019). Paternal age at
257 conception (i.e. PAC) and maternal age at conception (i.e. MAC) effects were analysed in general linear
258 mixed models (GLMMs), with RLTL measurements square-root transformed to meet assumptions of

Gaussian error distributions, and subsequently turned into Z-scores (Verhulst, 2020). We checked fixed effects for collinearity through variance inflation factors (VIF < 3).

We first determined the correlation between PAC and MAC to investigate whether analyses for PAC and MAC effects needed to be conducted separately. There were 471 RLTL measurements from 240 offspring (121 females and 119 males; with 108 unique fathers and 120 unique mothers) where MAC and PAC were known. PAC and MAC both spanned ages 1–12 years and there was a weak positive correlation between PAC and MAC (Pearson's $r = 0.160$, $P < 0.001$; Figure S1), allowing for PAC and MAC effects to be tested in the same model.

The effects of PAC and MAC on offspring RLTL were subsequently tested using linear mixed effect models in *lme4* 1.1–14 (Bates, Machler, Bolker, & Walker, 2015). The model included fixed covariates for the best-fitting age relationship with RLTL, which was a threshold model (van Lieshout et al., 2019), and a fixed factor for season. Individual ID, cohort (i.e. birth year), year, qPCR plate, row on qPCR plate, maternal ID, paternal ID and social group were included as random effects. MAC and PAC were added to this model as fixed effects, and their interaction with sex, where significance was tested using parametric bootstrapping ($n = 5000$ iterations; 471 measurements; 240 badgers). When interactions with sex were non-significant we re-ran the model without the interaction to test first-order effects.

Based on our dataset and model structure, we have ≥80% statistical power to detect a PAC effect of ≥0.00067 (Figure S2) using a simulation-based power analysis in *simr* 1.0.5 (Green & MacLeod, 2016). This is equivalent to a correlation coefficient of ≥0.131 (with the PAC effect size multiplied by its standard deviation and divided by the standard deviation of RLTL; Froy et al., 2017), providing statistical power to detect correlation coefficients found previously in humans (r = 0.127–0.160; de Meyer et al., 2007; Eisenberg et al., 2017; Nordfjall et al., 2010) and chimpanzees (r = 0.378; Eisenberg et al., 2017). While more complex relationships between PAC, MAC and RLTL may exist, for example threshold and non-linear associations, as seen in this badger population between leukocyte RLTL and age, we did not see evidence of this from visual inspection of the raw data (Figure 1), plus

285 the sample size is relatively small to test for more complex relationships, so we have not investigated
286 these.

287 Additional models were run, where only offspring RLTL measurements from cubs (<1 year old)
288 were included, to ensure the inclusion of adults did not mask effects of PAC or MAC. There were 194
289 measurements from 194 cubs (94 females, 100 males) that had 97 unique fathers and 109 unique
290 mothers. The cub model was similar to the full model, but did not include random effects for individual
291 ID (i.e. no repeat measures) and year (i.e. equivalent to cohort).

292 We then separated, including all offspring RLTL measurements, within- from between-
293 parental effects (*n* = 471 measurements; 240 badgers) for each parent to test for longitudinal PAC and
294 MAC effects, by taking the mean age that each parent conceived offspring at (between-parent effect)
295 and subtracting this mean from each of the ages that the parent conceived offspring at (within-parent
296 effect; van de Pol & Wright, 2009). Age at conception was estimated as the integer age in years of
297 when the parent conceived offspring, as due to delayed implantation conception can occur from
298 February until implantation occurs in December (Yamaguchi et al., 2006).

299

300 2.4.2 *Partitioning variance in RLTL*

301 We determined the relative contribution of environmental and genetic components to variation in
302 RLTL with a quantitative genetic 'animal model', using pedigree relatedness based on parent–offspring
303 assignments (*n* = 1248 measurements; 612 badgers). We had ≥80% power to detect a heritability of
304 RLTL of ≥0.27 (Figure S3), estimated using *pedantics* 1.7 (Morrissey & Wilson, 2010). We used a
305 stepwise addition approach to facilitate the detection of confounding random effects (Charmantier et
306 al., 2014), while estimating the changes in heritability in response to addition of random effects.
307 Additionally, we present results without fixed effects, as random effects are conditioned on the fixed
308 effects (Wilson, 2008).

309 We used *MCMCglmm* 2.25 (Hadfield 2010), with the number of iterations set to 600,000, a
310 thinning of 300 and burn-in period of 15,000 iterations. The response variable was untransformed

311 RLTL to gain variance estimates on the scale the trait was measured on (de Villemereuil, Schielzeth,
312 Nakagawa, & Morrissey, 2016); only a square-root tranformation of RLTL met Gaussian assumptions,
313 however, a square-root link is not available in *MCMCglmm*. Three thresholds of age at measurement
314 (van Lieshout et al., 2019) were included as fixed covariates and season as a fixed factor. The random
315 effects included: additive genetic, permanent environment (to account for environmental and non-
316 additive genetic between-individual variation), parental effects (mother and father ID), year effects
317 (cohort and capture years), resident social group, and measurement effects (qPCR plate and row, to
318 account for variance generated during the laboratory analysis).

319 We present results with qPCR plate and row included and excluded from the total phenotypic
320 variance when calculating heritability, since qPCR plate and row represent technical, not biological,
321 variance (de Villemereuil, Morrissey, Nakagawa, & Schielzeth, 2018). Additionally, since *MCMCglmm*
322 treats individuals with no parents assigned as founders (Hadfield, 2010), they will be assumed to be
323 unrelated despite potentially being related to each other in this population. We therefore confirmed
324 that our conclusions remained unchanged when these 159 offspring with no mother or father
325 assigned, were removed from the pedigree (Table S2; Model 8).

326 Since badgers exhibit increases as well as decreases in RLTL in later life, and juvenile RLTL (≤29
327 months old) does not vary with age cross-sectionally (van Lieshout et al., 2019), we also estimated
328 variance components and heritability just using a dataset of juvenille RLTL (≤29 months old; n = 837
329 measurements; 556 badgers). We had ≥80% power to detect a heritability of ≥0.28 (Figure S4). The
330 random effects were the same as in the full dataset. For the fixed effects the difference was that age
331 was included as a linear covariate rather than a threshold model (as the first threshold is at 29 months;
332 van Lieshout et al., 2019).

333 For random effects we used parameter expanded priors (F distribution: V = 1, nu = 1, alpha.mu
334 = 0, alpha.V = 1,000) since variance components were close to zero. Model convergence was checked
335 through low autocorrelation between successive thinned samples (<0.1), Heidelberg and Welch's
336 diagnostic (to see if samples are drawn from stationary distribution), Geweke diagnostic (equality of

means of first 10% and last 50% of Markov chain), and whether the effective size was >1000 for both fixed and variance components. Fixed effects were considered significant if the 95% credibility intervals of the posterior mode did not overlap zero.

We also conducted an analysis in *ASReml-R* 3 using the same model structure to determine the robustness of our variance component estimates given their dependency on the selected Bayesian prior. In *ASReml-R*, the significance of fixed effects was determined through Wald Z tests, whereas significance of random effects was determined through twice the difference in log-likelihood (Visscher, 2006).

Finally, we estimated evolvability, additive genetic variance divided by the squared trait's mean ($I_A = V_A$ / trait mean^2), for all individuals, and for juveniles only.

3. Results

Neither maternal age at conception (i.e. MAC) nor paternal age at conception (i.e. PAC) showed an overall, or offspring sex-specific, association with variation in offspring RLTL at any age (Figure 1a & 1b, respectively, and Table S3 & S4), or as cubs (Figure 1c & 1d, respectively; Table S5 & S6). Additionally, within- and between-parental age at conception effects for each parent were not linked to variation in offspring RLTL (Table S7).

The additive genetic variance explained near zero of the total phenotypic variance in RLTL (Table S2, Models 1–9). Heritability (h^2) was < 0.001 (95% CrI = <0.001–0.026) with qPCR plate and row variance included in the phenotypic variance (Table S2, Model 7) and 0.001 (95% CrI = <0.001–0.028) when qPCR plate and row variance were excluded. In contrast, year (with technical variance included: 0.251, 95% CrI = 0.143–0.459; and excluded: 0.321, 95% CrI = 0.155–0.483) and cohort (0.030, 95% CrI = 0.007–0.074; 0.035, 95% CrI = 0.007–0.079) explained a greater proportion of the phenotypic variance in RLTL (Figure 2; Table S2, Model 7). Social group (with technical variance included: <0.001, 95% CrI = <0.001–0.014; and excluded: <0.001, 95% CrI = <0.001–0.016), paternal (<0.001, 95% CrI =

<0.001–0.025; <0.001, 95% CrI = <0.001–0.026) and maternal (<0.001, 95% CrI = <0.001–0.030; <0.001, 95% CrI = <0.001–0.033) effects explained near zero variance in RLTL (Figure 2; Table S2, Model 7).

There was also no detectable heritability of juvenile RLTL (≤29 months old; with technical variance included; h^2 <0.001, 95% CrI = <0.001–0.043), moderate year (0.216, 95% CrI = 0.107–0.431) and small cohort (0.037, 95% CrI = 0.003–0.123) effects, and no detectable social group (<0.001, 95% CrI = <0.001–0.020), paternal (<0.001, 95% CrI = <0.001–0.026) or maternal (<0.001, 95% CrI = <0.001–0.032) effects (Table S2, Model 9).

A frequentist approach in *ASReml–R* showed similar results with additive genetic variance explaining near zero of the phenotypic variance, but with cohort and year effects explaining variation in RLTL (Table S8 & S9).

Evolvability of RLTL was <0.001 (95% CrI = <0.001–0.005) including all individuals (model 7) and was <0.001 (95% CrI = <0.001–0.007) for juveniles only (model 9).

4. Discussion

4.1 Parental age at conception effects

Our study found no evidence for paternal age at conception (i.e. PAC) or maternal age at conception (i.e. MAC) associations with offspring RLTL in this European badger population. Studies in vertebrates have provided evidence for positive (e.g. Eisenberg et al., 2017; Kimura et al., 2008; Njajou et al., 2007), negative (summarised in Table 1 in Belmaker et al., 2019; Eisenberg, 2019) or no (summarised in Table 1 in Eisenberg, 2019) PAC effect, and positive (Asghar et al., 2015) or no (Bauch et al., 2019; Belmaker et al., 2019; Bouwhuis et al., 2018; Froy et al., 2017; Heidinger et al., 2016; McLennan et al., 2018) MAC effect on offspring telomere length. In cross-sectional mammalian studies, positive PAC effects have been reported in humans, a negative PAC effect was found in a captive population of short-lived house mice (*Mus musculus*; de Frutos et al., 2016), and in a wild population of longer-lived Soay sheep there was no relationship between offspring RLTL (either measured across all ages or only as lambs) and PAC or MAC (Froy et al., 2017). Five non mutually-exclusive explanations for positive,

negative and no PAC effects in mammals are: 1) variation in lifespan between study populations, with a negative effect in a short-lived mammal (de Frutos et al., 2016), and positive or no PAC effects in longer-lived mammals (e.g. Eisenberg et al., 2017; Froy et al., 2017; this study; Kimura et al., 2008). 2) differences in mating systems and associated sperm production rates, with positive PAC effects in species with higher sperm production rates due to greater telomere lengthening or more selective loss of germ stem cells with shorter telomeres (Bouwhuis et al., 2018; Froy et al., 2017). 3) masking by sex-specific effects on offspring, however, we tested for but did not detect these. 4) masking by selective disappearance of poor quality parents from the population, which was not the case in our study. 5) since a non-linear relationship between age and telomere length exists in badgers (van Lieshout et al., 2019) and Soay sheep (Fairlie et al., 2016) non-linear PAC/MAC effects may potentially be present. Although we did not statistically test for more complex ralationships due to our small sample size, visual inspection of the raw data did not show a non-linear relationship in our system (Figure 1) or Soay sheep (Froy et al., 2017).

Counter to our expectation for a highly promiscuous species that exhibits multiple and repetitive mounting behaviour (Dugdale, Griffiths, et al., 2011; Dugdale et al., 2007), we found no PAC effect, for which there are several potential reasons. First, telomerase activity may be more tightly regulated, or even lower, in the germline in badgers. However, while we know telomerase activity varies among tissue types and species (Davis & Kipling, 2005; Gomes et al., 2011), we require a better understanding of telomerase activity in species with different mating systems to validate this hypothesis. Secondly, higher sperm competiton and thus stronger selection on the male germline may reduce the variability in RLTL in male germ stem cells. If telomere lengths in the germline are more consistent, selective loss of germ stem cells with age will have a lower impact on mean telomere length in sperm and thus no subsequent PAC effect (Froy et al., 2017; Kimura et al., 2008). Thirdly, female badgers exhibit various postcopulatory mechanisms (i.e. embryonic diapause and superfoetation) which may obscure the relationship between PAC or MAC and offspring RLTL. Although cellular replication is suppressed during embryonic diapause, maternal stress could still

impact offspring RLTL through stress-related glucocorticoids (Angelier, Costantini, Blevin, & Chastel, 2018; Haussmann, Longenecker, Marchetto, Juliano, & Bowden, 2012; Yamaguchi et al., 2006). Alternatively, superfoetation could result in less exposure of the later fertilised zygote to maternal glucocorticoids. However, the effects of these postcopulatory mechanisms on PAC and MAC effects are difficult to quantify as we are unable to pinpoint conception and implantation dates. Finally, badgers have a much lower life expectancy than humans and chimpanzees (Bright Ross et al., 2020), as do Soay sheep (Froy et al., 2017). While reproductive senescence in badgers is observed in both sexes (Dugdale, Pope, Newman, Macdonald, & Burke, 2011; Sugianto, Newman, Macdonald, & Buesching, 2020), the effects of telomere elongation in sperm may not become apparent due to the shorter life expectancy of badgers, compared to humans and chimpanzees.

Even though in male badgers the testes ascend in autumn with no spermatogenesis (Sugianto et al., 2019), sperm production is likely highest in the peak mating season immediately after parturition (Macdonald et al., 2015). Despite the potential for sperm competition in badgers, the seasonal mating peaks may explain the lack of a PAC effect due to the lack of continuity and rate of sperm production, as recently hypothesised in Bouwhuis et al. (2018).

Non-linear relationships observed between age and RLTL may also occur between age and sperm telomere length, leading to non-linear PAC effects. For example, when there is a correlation between sperm and leukocyte telomere length, as seen in humans (Ferlin et al., 2013), a non-linear PAC effect is expected. However, the presence and direction of non-linear, linear or no PAC effect may depend upon the level of telomerase activity in the testes, and the degree of germ stem cell selection on telomere length (Hjelmborg et al., 2015; Kimura et al., 2008). While sperm are produced throughout life, oocytes are in place at birth and therefore linear MAC effects are predicted if oocyte quality varies and higher-quality occytes are used earlier in life (Monaghan et al., 2020), or alternatively no MAC effect may occur. PAC and MAC effects are less consistent in wild populations than in humans, and the underlying mechanisms may entail more than just the degree of promiscuity in a system.

4.2 Heritability of telomere length

While our study reveals no heritability of RLTL, we did not have the statistical power to detect heritability of RLTL <0.27. The low power may be attributable to the pedigree structure, in terms of a realtively low number of full-sibs (Table S1), due to multiple paternity within litters and high extra-group paternity in badgers (Annavi, Newman, Dugdale, et al., 2014; Dugdale et al., 2007), and a low mean pairwise relatedness (Table S1). Given that the variance in RLTL explained by individual identity was very low at 2%, which forms the upper limit to ordinary narrow-sense heritability, the contribution of additive genetic variance to total phenotypic variance in RLTL in this wild mammal population is low. The low heritability of RLTL is consistent with low heritability of fitness-related traits in other species (Kruuk et al., 2000; Teplitsky, Mills, Yarrall, & Merila, 2009). Additionally, we found low evolvability of RLTL and thus little potential for evolutionary change under selection (Hansen, Pélabon, & Houle, 2011). We have previously identified associations between early-life RLTL (<1 year old) and survival probability in badgers (van Lieshout et al., 2019), so selection may have eroded genetic variation underlying RLTL in this population (Mousseau & Roff, 1987; Postma, 2014; Price & Schluter, 1991). Our study however contrasts with human studies that estimate higher heritability of telomere length (summarised in Table 1 in Dugdale & Richardson, 2018), although these studies could not separate additive genetic effects from shared environments either because parent-offspring regressions were used or because environmental risk factors were included as covariates rather than random effects.

Partitioning of variation in RLTL in badgers into genetic and environmental factors showed that variation in RLTL was largely driven by environmental variation. Of the environmental factors investigated, we found no evidence for social group, maternal or paternal effects explaining variation in RLTL. Even though nest or social group (Becker et al., 2015; Boonekamp et al., 2014; Cram et al., 2017; Nettle et al., 2015) and maternal effects (Asghar et al., 2015) are important effects on telomere length variation in other species, this is not the case for our badger population. Badger mothers

provide neonatal care up to independence at around 14–16 weeks (Dugdale, Ellwood, & Macdonald, 2010; Fell, Buesching, & Macdonald, 2006), and we therefore cannot capture badgers until at least 3 months of age (Protection of Badgers Act, 1992). As the strength of maternal effects on offspring decline with the age of the offspring (Moore, Whiteman, & Martin, 2019), maternal effects explaining variation in offspring RLTL become more difficult to detect. While changing leukocyte ratios with age may drive within-individual changes in telomere length, we have found evidence that leukocyte cell composition changes with age in males but not females (van Lieshout et al., 2020). Even though human and baboon lymphocytes have shorter telomeres than neutrophils (Baerlocher, Rice, Vulto, & Lansdorp, 2007; Kimura et al., 2010), variation in leukocyte telomere length in Soay sheep did not influence variation in telomere length (Watson et al., 2017). Since there is no sex difference in telomere length across ages in our study population (van Lieshout et al., 2019), a change in leukocyte cell composition is unlikely to contribute to variation in telomere length.

We found a small effect of cohort on RLTL which is in accordance with previous studies in mammals and birds which had shorter telomeres, or accelerated telomere shortening, when subject to sub-optimal natal conditions (Fairlie et al., 2016; Hall et al., 2004; Nettle et al., 2015; Watson et al., 2015). However, the variance explained by the year in which the individual was captured was about eight times greater than the cohort effect, even though we could not separate cohort and year effects for 163 badgers since they died as cubs. Although we cannot identify the specific drivers of the association between year and variation in RLTL, badgers are sensitive to annual weather variation (Macdonald et al., 2010; Nouvellet et al., 2013), which affects their food availability, and can lead to elevated levels of oxidative stress (Bilham et al., 2018). Additionally, exposure to diseases may vary among years and could contribute to variation in RLTL (Newman, Macdonald, & Anwar, 2001; Sin et al., 2014). Furthermore, the size of the extant population increased substantially over the study interval (with no change in range), causing considerable inter-annual variation in population density (Bright Ross et al., 2020; Macdonald & Newman, 2002; Macdonald et al., 2009) that could lead to RLTL variation in badgers.

Since an evolutionary response depends on the magnitude of both natural selection and the heritability of the trait (Kruuk 2004; Lynch & Walsh 1998), the evolutionary potential of telomere length, in this badger population, appears to be low. Instead, variation in badger RLTL is largely driven by non-additive genetic sources such as variation between cohorts and years. Further research is required to understand which and how specific environmental and social factors impact an individual's physiology and contribute to variation in RLTL.

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

This study was conceived by S.H.J.v.L., A.B. and H.L.D., and developed by A.M.S.; 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 the telomere laboratory

work with advice from T.B. and statistical analyses with input from A.M.S. and H.L.D.; The paper was written by S.H.J.v.L. and H.L.D. and all authors contributed critically and gave final approval for publication.

Data accessibility

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

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Figures and tables

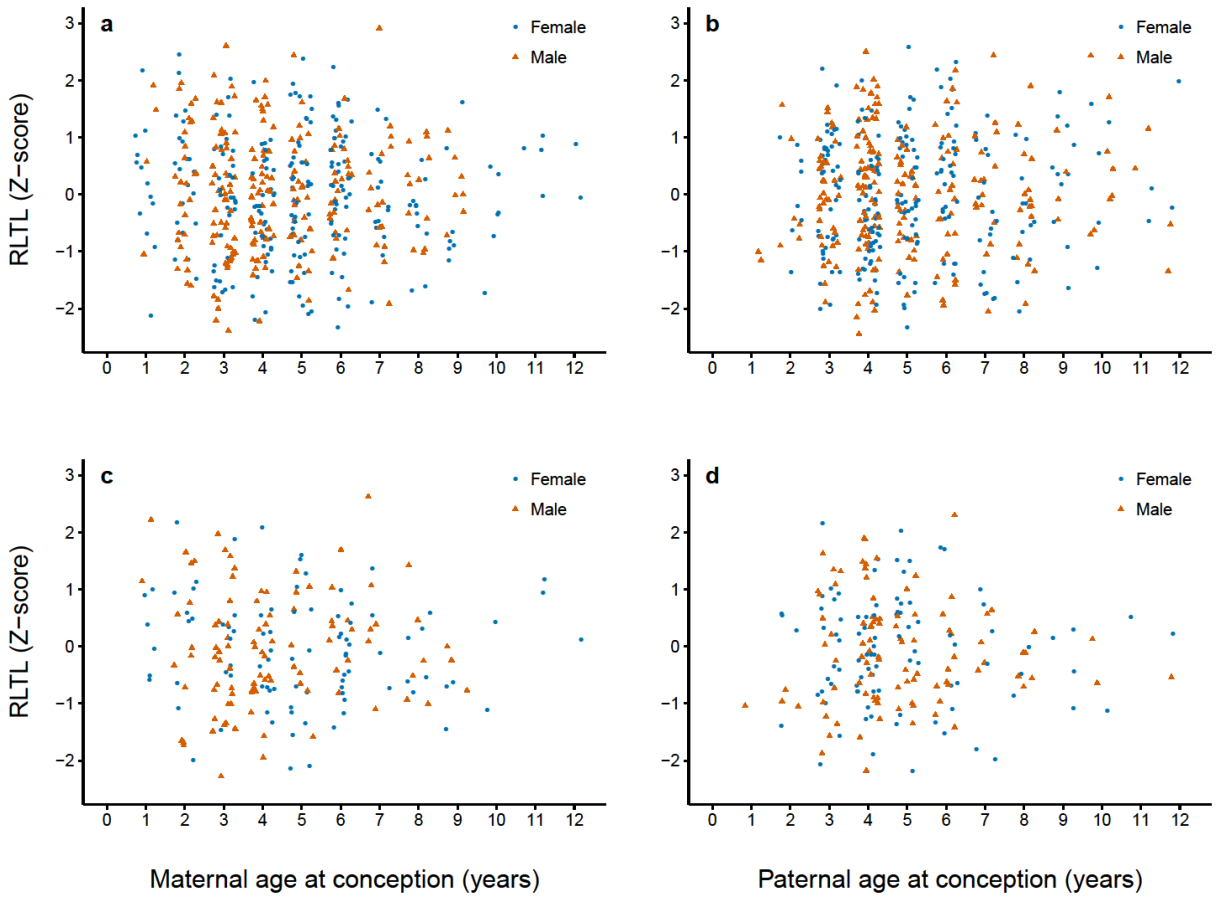
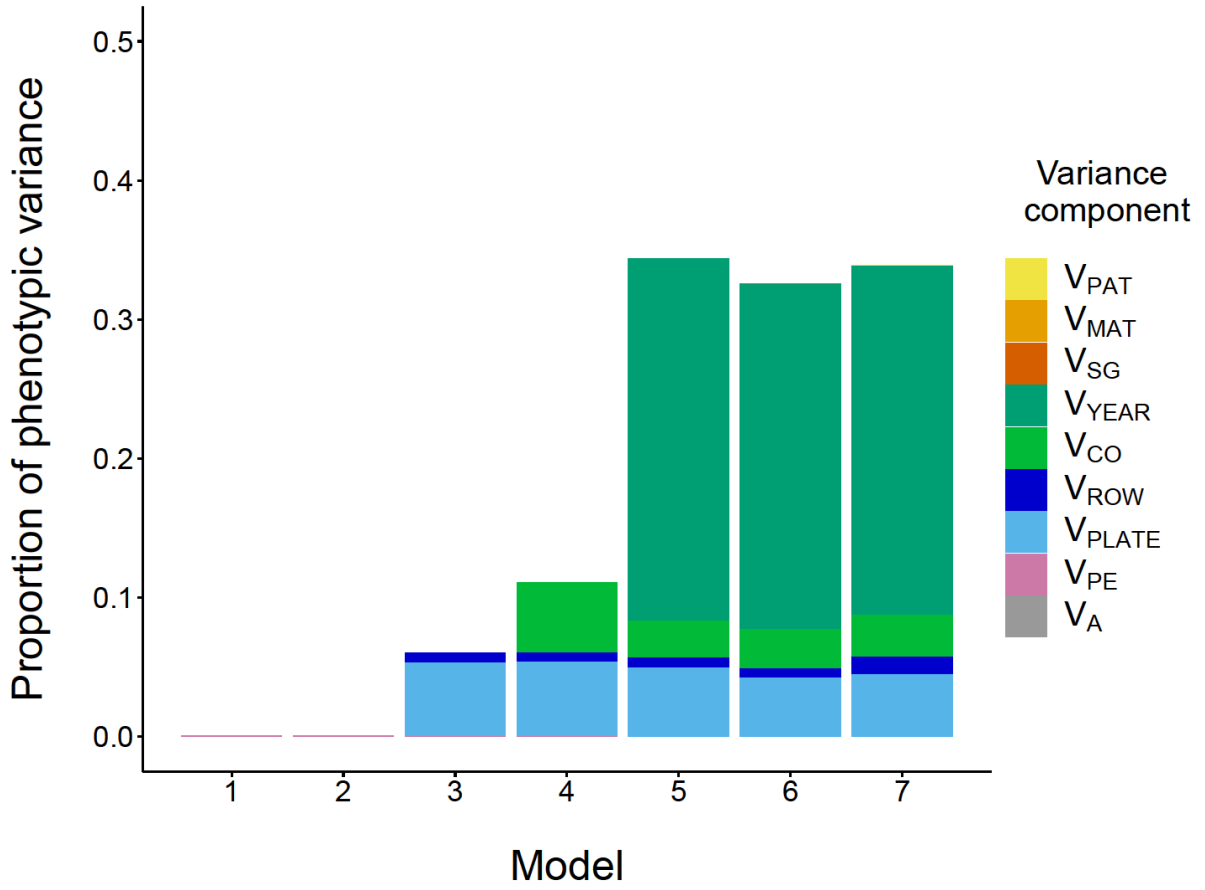


Figure 1 Associations between offspring relative leukocyte telomere length (RLTL) and either maternal (a & c) or paternal (b & d) age at conception (years) in European badgers. Scatterplots show raw data (blue for females and brown for males) for all ages (a & b; *n* = 417 measurements; 240 badgers) or only offspring measured as cubs (<1 year; c & d; 194 measurements; 194 badgers), and jittered for clarity.



927
928 **Figure 2** Proportion of variance explained in relative leukocyte telomere length (RLTL; models 1–8) in
929 European badgers of all ages. Variance components: V_A = additive genetic, V_{PE} = permanent
930 environment, V_{PLATE} = plate, V_{ROW} = row, V_{CO} = cohort, V_{YEAR} = year, V_{SG} = social group, V_{MAT} = maternal,
931 and V_{PAT} = paternal. Model numbers on the x-axis correspond with Table S2.