Telomere dynamics in female Columbian ground squirrels: recovery after emergence and loss after reproduction

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2   **squirrels: recovery after emergence and loss after reproduction**

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17 **Author contribution statement**

18 VAV, FSD and FC designed the study. VAV, SS, CS, JFR, QS and FSD collected the data in

19 the field. VAV and CS performed the statistical analysis and ML and SZ the laboratory

20 analyses and check the quality of the qPCR results with FC. VAV and FC wrote the first draft

21 of the manuscript. ML and SZ wrote sections of the manuscript. All authors contributed to

22 manuscript revision, read and approved the submitted version.

23

24

ABSTRACT

Telomeres are specialized non-coding DNA sequences at the end of chromosomes that protect genetic information. Telomere loss over lifespan is generally viewed as a phenomenon associated with ageing in animals. Recently, telomere elongation after hibernation has been described in several mammals. Whether this pattern is an adaptation to repair DNA damage caused during rewarming from torpor or if it coevolved as a mechanism to promote somatic maintenance in preparation for the upcoming reproductive effort remains unclear. In a longitudinal study measuring telomere length using buccal swabs, we tested if telomere elongation was related to reproductive success in wild adult female Columbian ground squirrels (*Urocitellus columbianus*) that were monitored from emergence from hibernation to the end of the reproductive season. We found three key results. First, female telomere length increased at the start of the breeding season, both in breeding and non-breeding individuals. Second, post-emergence telomere lengthening was unrelated to female future reproductive output. Third, telomere length decreased in breeding females during lactation, but remained stable in non-breeding females over a similar period. Within breeders, telomeres shortened more in females producing larger and heavier litters. We concluded that telomere lengthening after hibernation did not constrain immediate female reproductive capacities. It was more likely to be part of the body recovery process that takes place after hibernation. Telomere erosion that occurs after birth may constitute a physiological cost of female reproduction.

INTRODUCTION

Telomeres are sequences of non-coding DNA that cap the end of linear chromosomes and protect the integrity of coding DNA where key genetic information resides (Greider, 1991). As cells replicate, telomeres progressively shorten due to the end-replication problem of DNA (viz., the extremities of DNA on the lagging-strand are lost due to a lack of template) (Harley, Futcher, & Greider, 1990; Watson, 1972). Although part of this replication problem is counterbalanced by telomerase, an enzyme that rebuilds otherwise dwindling telomeres (Greider & Blackburn, 1985), critically short telomeres lead to cells leaving the normal replicative cycle through cell apoptosis (Blackburn, 1991). From an organismal perspective, telomere length, and maybe more so the rate of telomere loss, are increasingly considered reliable proxies of individual or species lifespan, both in captive (*e.g.* Heidinger et al., 2012; Whittemore et al., 2019) and free-living vertebrates (reviewed by Wilbourn et al. 2018). Telomeres are associated with individual fitness in numerous species (Bichet et al., 2020; Dupoué et al., 2017; van Lieshout et al., 2019).

Beside the end-replication problem, a growing number of studies have highlighted how telomere length may be an integrative proxy of individual stress, as telomere length integrates the accumulation of stress-related damages with age, and thus reflects individual life experiences (Dupoué et al., 2020; Monaghan & Haussmann, 2006). For instance, environmental harshness (*e.g.* pollution levels, disease prevalence, oxidative stress, environmental and social stressors; reviewed by Chatelain, Drobniak, & Szulkin, 2020) has been shown to negatively affect telomere length in groups as diverse as fish (Molbert et al., 2021), birds (Asghar et al., 2015; Aydinonat et al., 2014; Grunst et al., 2020), and mammals (Kesäniemi et al., 2019), including humans (Blackburn & Epel, 2012). As data grow, so does our understanding of the importance of these highly conserved DNA structures in shaping life-history trade-offs and tactics across species (Dantzer & Fletcher, 2015; Young, 2018).

71   Telomere characteristics are likely to shed useful mechanistic insights on life histories.

72   This is because telomere loss may reflect the physiological costs that an individual has to pay

73   when limited energy is invested in potentially competing, resource-demanding life-history

74   functions (such as reproduction and self-maintenance). This is based on the hypothesis that

75   telomere maintenance is an energetically costly mechanism (Young, 2018). Whereas we have

76   no direct evidence for this hypothesis so far, indirect evidence has been accumulating from

77   studies focusing on reproductive costs and from hibernating species (*e.g.* Hoelzl et al., 2016;

78   Nowack et al., 2019).

79   In fact, experimental increases in long-term reproductive effort and actuarial

80   senescence (increased parental mortality rates) have been positively correlated (Boonekamp,

81   Bauch, & Verhulst, 2020), suggesting that reproduction somehow triggers ageing mechanisms

82   in adults (Flatt & Heyland, 2011). Experimentally increasing reproductive effort was found to

83   hasten telomere shortening of breeding birds (Reichert et al., 2014; Sudyka et al., 2014; for

84   contrasting results see Sudyka et al., 2019). While most experimental studies published to date

85   supported the hypothesis of accelerated telomere erosion as a cost of reproduction, negative

86   associations between reproductive traits and telomere dynamics were only found in roughly

87   half of the studies conducted in wild vertebrates (Sudyka et al. 2019). Thus, whether the

88   negative impact of reproduction on telomeres is a general phenomenon across vertebrates

89   remains to be determined (*e.g.* Sudyka et al. 2016; Sudyka et al., 2019), and further evidence

90   is needed, particularly in non-avian species.

91   Previous studies on hibernating rodents convincingly demonstrate that telomere

92   maintenance is achieved at energetic costs. Hibernation is composed of periods of decreased

93   body temperature (torpor) and rewarming events, and the frequency of these torpor events or

94   rewarming events and associated metabolic activation have been related to telomere dynamics

95   (Turbill et al., 2012; Turbill et al., 2013; Hoelzl et al. 2016). Interestingly, experimental

96   manipulation of food supply or of ambient temperature during or after hibernation were shown

97   to impact both telomere shortening or elongation, suggesting an energy cost of telomere

98   maintenance (Hoelzl et al. 2016, Nowack et al. 2019). In any case, hibernating species appear

99   to retain the seasonal ability to reconstruct their telomeres after hibernation (*i.e.* at the start of

100 the active season, Hoelzl et al., 2016; Turbill et al., 2012; Turbill et al., 2013). Such a

101 phenomenon may characterize the switch of adult physiology from a non-active hibernating

102 state to an active reproductive state (Hoelzl et al., 2016), and has been suggested as an

103 immediate post-hibernating and pre-reproductive strategy (Nowack et al., 2019), but its actual

104 benefits in terms of fitness remain unclear. Telomere elongation at emergence from hibernation

105 could occur as part of a somatic maintenance process related to hibernation (Turbill, Bieber, &

106 Ruf, 2011), as an anticipatory process in order to buffer the oxidative (telomere-shortening)

107 costs of upcoming reproduction (*i.e.* oxidative shielding hypothesis; Blount et al. 2016, Viblanc

108 et al. 2018), or both. Teasing apart those alternatives requires information from hibernating

109 species in which seasonal telomere dynamics at the individual level are assessed at emergence

110 from hibernation and then longitudinally throughout the breeding season. By comparing

111 breeding and non-breeding individuals sampled at similar time periods, it is possible to test

112 whether telomere length, telomere lengthening or telomere loss predict individual reproductive

113 success, or reflect a cost of reproduction, while controlling for individual differences in age

114 and condition (*i.e.,* inter-individual differences in body mass at the onset of the breeding

115 season).

116   We tested the hypothesis that telomere dynamics (both lengthening and shortening)

117 varied as a response to hibernation and reproduction in free-living female Columbian ground

118 squirrel (*Urocitellus columbianus*). Columbian ground squirrels provide a robust model system

119 for addressing these questions. These montane rodents have an especially short active season

120 (3-4 months) during which females emerge, soon mate, gestate (ca. 24 days), lactate and raise

121   their young (ca. 27 days), molt and fatten before subsequent hibernation (Dobson, Badry, &

122   Geddes, 1992; Dobson & Murie, 1987). The reproductive season starts shortly after emergence

123   from hibernation, and females typically enter oestrus 3-4 days following emergence (Lane et

124   al. 2011). Thus, this is a short and highly active period following a long period of metabolic

125   inactivity. For females, lactation is an energy-demanding period when oxidative metabolism is

126   high (Skibiel, Speakman, & Hood, 2013; Speakman, 2008) and when the oxidative costs of

127   reproduction are expected to be highest. Interestingly, these females seem to have evolved

128   increased antioxidant defenses, which are highest during lactation (Viblanc et al. 2018),

129   allowing them to buffer the oxidative costs of reproduction (*i.e.* the oxidative shielding

130   hypothesis; Blount et al. 2016). This suggest that lactation entails physiological costs, but

131   whether those costs extend to damage to telomeres remains to be tested.

132   We hypothesized that, at the start of the breeding season (from emergence of

133   hibernation to parturition), an increase in telomere length might be expected both with regards

134   to somatic maintenance/reconstruction following hibernation and as part of a mechanistic

135   process that buffers the later oxidative costs of lactation (Blount et al., 2016). On one hand,

136   and after controlling for individual age and condition, a positive association between starting

137   telomere length and reproductive output would suggest that telomere length acts as a constraint

138   on reproduction (*i.e.,* females with low somatic condition would not invest in reproduction).

139   On the other, a positive relationship between reproductive output and telomere loss over

140   lactation would suggest that reproductive effort indeed entails physiological costs (*i.e.,* females

141   with high reproductive outputs exhibit greater reduction in telomere length over the breeding

142   season). To test this, we followed known-aged females at emergence from hibernation and over

143   the course of the breeding season. Since nothing is known so far about how variable telomere

144   length is in this species, or whether telomere length is related to individual age or body

145   condition, we first evaluated the individual repeatability of telomere length over the breeding

season, and the potential effect of female age and condition (mass) on telomere length. Then, we evaluated: (i) if female telomere lengthening occurred at the start of the breeding season, from emergence of hibernation to parturition; (ii) if telomere length at emergence from hibernation (and potential subsequent reconstruction from emergence to parturition) predicted female reproductive output (assessed by litter size and mass measured at weaning); and (iii) if female reproductive output was associated with increased telomere loss during lactation.

METHODS

Study site and population monitoring

Female Columbian ground squirrels were monitored in a 2.6 ha subalpine meadow from 1992 to 2019 in the Sheep River Provincial Park, Alberta, Canada (50 38' 10.73" N; 114° 39' 56.52"W; 1520 m), as part of a long-term study on their ecology, behavior, and physiology (Dobson et al. 2020). Reproduction takes place over the short summer season (3-4 months), during which sexually mature females (2-14 years old) produce a single litter (2-7 pups) over 51 days (24 days of gestation and *ca* 27 days of lactation and post-weaning parental care, Murie and Harris 1982). In each year, the entire population was trapped at emergence from hibernation, using 13 x 13 x 40 cm traps (Tomahawk Live Traps; Hazelhurst, WI, USA) baited with a small amount of peanut butter. Upon trapping, each ground squirrel was weighed to the nearest 5 g (Pesola spring-slide scale; Pesola® Ag; Baar; Switzerland), tagged if unmarked (#1-Monel metal tag; National Band and Tag Company, Newport; KY) and painted with a unique dorsal mark for visual identification with black hair dye (Clairol, Stamford, CT). Consequently, all females in the population were of known age. Buccal tissue for telomere length (see below) was collected during the 2019 active period.

We observed animals daily (from ~ 7:00 am to 2:00 pm), starting at emergence from hibernation and continuing throughout the reproductive season. We determined mating dates

171 for each female from male-female interactions, the occurrence of below-ground consortships

172 or above-ground mating events (Raveh et al., 2010, 2011). All females were surveyed daily

173 until we were certain that they had (or had not) reproduced, as confirmed by mating behavior.

174 When in doubt, we inspected female genitalia for the presence of copulatory plug material and

175 dried sperm as indicators of successful mating events (Murie & Harris, 1982). We flagged the

176 location of female nest-burrows (single-entrance burrows where females raise and lactate to

177 feed their young), identified by daily observations of female's morning emergences from these

178 burrows and observations of females stocking them with loads of dry grass. Mothers and their

179 entire litters were trapped at about the time of weaning, when offspring first emerged above

180 ground, approximately 51 days after mating and 27 days after parturition. All offspring were

181 weighed to the nearest 1 g and given unique ear tags and dye markings.

182

183 **Tissue collection and telomere length measurement**

184 *Tissue collection*

185 Cell tissue was collected from the buccal mucosa of each ground squirrel by gently twirling a

186 Gynobrush® brush (Heinz Herenz Medizinalbedarf, Hamburg) on the inside of each cheek.

187 This technique is particularly adapted for repeated non-invasive sampling (Hoelzl et al. 2016),

188 and buccal cell DNA to reflect individual health status (e.g. in humans, Thomas et al. 2008)

189 The collected tissue was immediately transferred to 96% ethanol Eppendorf tubes and kept at

190 4°C until DNA extraction. For breeding females (N = 19; age = 4.4 ± 1.9 years old, range = 2

191 – 8 years old), tissue was collected at four points calculated for each individual: emergence

192 from hibernation (or the day after), and then 26, 38 and 54 days later (around the times of

193 parturition, mid-lactation, and offspring weaning). Tissue was collected from non-breeding

194 females (non-breeding females: N = 24; age = 2.6 ± 2.0 years old, range = 1 – 7 years old)

195 following a similar schedule. Overall, we were able to acquire tissue for 43 females including

196   19 breeding and 24 non-breeding individuals all sampled four times (i.e. 172 samples).

197   However, differences in sample sizes between our different analyses are due to varying success

198   at acquiring samples in the field or telomere amplification in the laboratory, or missing data in

199   other parameters on some occasions (*e.g.,* female mass).

200

201   *DNA extraction*

202   DNA extraction was carried out using the Nucleospin Tissue kit, Macherey-Nagel, Düren,

203   Germany, and checked for quality using gel-migration (DNA integrity) and a NanoDrop 1000

204   (ThermoFisher Scientific, Waltham, MA, USA) spectrophotometer (absorbance ratio

205   A260/280; A260/230, DNA quality). Briefly, after allowing ethanol to evaporate from the

206   tissue sample, lysis was achieved by incubation of the sample material in a proteinase K / SDS

207   solution. Appropriate conditions for DNA binding to the silica membrane in the NucleoSpin®

208   Tissue Columns were achieved by adding chaotropic salts and ethanol to the lysate. The

209   binding process was reversible and specific to nucleic acids. Contaminants were removed by

210   subsequent washing with two different buffers as indicated by the kit's protocol. Pure genomic

211   DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.

212

213   *Telomere qPCR amplification*

214   Extracted DNA was used to amplify both the telomere sequence and a control gene by

215   quantitative real-time amplification (qPCR) based on Cawthon's (2002) original publication.

216   As a reference gene that was invariant in copy number (non-VCN, Smith et al. 2011), we used

217   a 54 bp portion of the c-myc proto-oncogene, which was tested for non-variability in copy

218   number in our population using amplicon gel migration (see Online Supplementary Materials).

219   Forward and reverse telomeric primers were 5'- CGG TTT GTT TGG GTT TGG GTT TGG

220   GTT TGG GTT TGG GTT - 3' and 5'- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC

221   CCT TAC CCT - 3'. Primer sequences for the non-VCN gene were 5'- GAG GGC CAA GTT

222   GGA CAG TG - 3', and 5' - TTG CGG TTG TTG CTG ATC TG -3'. A master mix was

223   prepared for each primer set containing 5 µL GoTaq® qPCR Master Mix (Promega, Madison,

224   WI, USA).

225   We used telomere primers at a concentration of 200 nM and non-VCN primers at 400

226   nM in a 10 µL reaction. Each sample of DNA was diluted to 2.5 ng/µL with double-distilled

227   H_2O just prior to running the reactions, and 2 µL of this sample is used in each 10 µL reaction.

228   qPCR conditions for telomeres were 2 min at 95°C followed by 30 cycles of 15 s at 95°C, 30

229   s at 56°C and 30 s at 72°C. qPCR conditions for the non-VCN gene were 2 min at 95°C

230   followed by 40 cycles of 15 s at 95°C, 30 s at 59°C and 1 min at 72°C. Amplifications were

231   done on a 384-well thermocycler (CFX-384 Touch Real-Time PCR Detection System, Biorad,

232   USA). Duplicates of each sample's telomere and non-VCN qPCR amplifications were

233   performed on separate plates (*i.e.* forming a qPCR run), the amplification conditions being

234   different between telomere and non-VCN sequences (see above). In total, all samples of our

235   experiment were measured over 2 runs.

236   In addition to our ground squirrel samples, all plates included a no template control

237   (water) and a 'calibrator' sample in duplicate. The calibrator was DNA extracted from a single

238   individual (golden sample randomly chosen among those for which a large quantity of DNA

239   was available), diluted to the same concentration as other ground squirrel samples (2.5 ng/µL).

240   Both a negative control (water) and melting curves were run for each plate to control for the

241   absence of (i) non-specific amplification and of (ii) primer-dimer artefact. On each plate, we

242   also included a calibrator sample dilution curve (from 10 ng/µL to 0.3125 ng/µL) to evaluate

243   plate amplification efficiencies of the telomere sequence and the non-VCN gene, and to check

244   that the Cq values produced declined in a log-linear fashion ($r^2 > 0.98$) before proceeding to

245   statistical analysis.

246   Among the amplification values of the control gene, we had 23 samples (13.4% of 172

247   samples,) which were delayed of 1 Cq compared to the mean Cq value. Running the analyses

248   with or without these samples yielded similar results, and thus we chose to keep them in the

249   present analysis. Efficiencies for the two reactions were 0.997 (range 0.996 – 0.999) for non-

250   VCN genes and 0.996 (range 0.991- 1.001) for telomeres. We calculated relative telomere

251   length (RTL) following Pfaffl (2001), as:

252
$$RTL = \frac{E_{TEL}{}^{Cq_{TEL[calibrator]}-Cq_{TEL[sample]}}}{E_{non-VCN}{}^{Cq_{non-VCN[calibrator]}-Cq_{non-VCN[sample]}}}$$

253   where E_{TEL} and $E_{non-VCN}$ are the mean plate efficiencies for each sequences, and $Cq_{TEL[calibrator]}$

254   and $Cq_{TEL[sample]}$ are the mean Cq for telomere calibrator and sample, respectively. Similarly,

255    $Cq_{non-VCN[calibrator]}$ and $Cq_{non-VCN[sample]}$ are the mean Cq for non-VCN calibrator and sample,

256   respectively. The intraclass correlation coefficient (ICC) was calculated for intra-run and inter-

257   run variation of T/S ratio following (Cicchetti 1994), and was recorded at 0.783 and 0.805,

258   respectively.

259

260   **Data analyses**

261   *Telomere length repeatability and relationship with female age and body mass*

262   First, we used a linear mixed model (LMM) approach to estimate individual repeatability in

263   telomere length over the course of a breeding season as

264
$$R = \frac{V_G}{V_P} = \frac{V_G}{V_G + V_R} \; ;$$

265   V_G is the among-individual variance in telomere length, V_P the total phenotypic variance in

266   telomere length, and V_R the within-individual (or residual) variance in telomere length

267   (Nakagawa and Schielzeth 2010, Stoffel et al. 2017). We accounted for potential error

268   measurement associated with qPCR assays by including assay-related terms (plate, row within

269   plate) as random factors in the model. Confidence intervals around repeatability estimates were

270 calculated by parametric bootstrapping (10,000 iterations) using the 'rptR' R cran package

271 (Stoffel et al., 2017). The statistical significance of repeatability estimates was calculated using

272 likelihood ratio tests, comparing the model fit to models where group-level variance was

273 constrained to zero.

274 Second, we ran a LMM ('lme4' package in R) to test whether female telomere length

275 was related to female age or condition (estimated as mass) at the time of measurement. For

276 this, female telomere length was specified as the dependent variable, and female age and mass

277 as independent variables. We included female ID as well as assay-related terms (plate, row

278 within plate) as random factors in the model to account for repeated measures on individual

279 females over the breeding season, and potential measurement error associated with qPCR

280 assays. Telomere length and independent variables were systematically standardized (z-scores)

281 prior to analyses to facilitate future comparisons between q-PCR-based telomere studies

282 (Verhulst, 2020), and so that estimates of models for independent variables could be directly

283 compared as effect sizes.

284

285 *Seasonal changes in telomere length*

286 Seasonal changes in telomere length were investigated separately in females using a LMM,

287 with telomere length specified as the dependent variable, and seasonal timing (emergence *vs.*

288 birth *vs.* lactation *vs.* weaning), breeding status (breeder *vs.* non-breeder), and their interaction

289 as independent variables. Breeding females were those observed lactating and raising a litter.

290 We specifically tested for the interaction between breeding status and seasonal timing, to test

291 whether seasonal dynamics in telomere length were different between breeding and non-

292 breeding females. Because our previous analyses showed significant influences of female age

293 and mass on telomere length, we controlled for those variables, as well as for female ID

294 (repeated measures over the season) and assay-related terms (plate, row within plate) as random

295   factors in the model. As above, all variables were standardized. As a measure of effect size, we

296   calculated percent changes in telomere length between seasonal time periods from marginal

297   means obtained from a similar LMM but where telomere length was not standardized (so that

298   actual percent differences between time periods could be calculated). Significant differences

299   between time periods were determined by Tukey Honest Significant Difference post-hoc tests

300   (HSD), which control for multiple testing, using the '*emmeans*' package. Marginal model

301   means are presented along with their 95% confidence intervals in the figures and significant

302   differences along with their 95% confidence intervals are presented in the tables.

303

304   *Telomere length in relation to subsequent reproduction*

305   For those females that bred, we tested whether telomere length at the start of the breeding

306   season predicted female reproductive output. Litter size (N = 18, litter size (± SD) = 2.5 ± 1.0,

307   range 1 – 5) or total litter mass (N = 18, total litter mass = 247.2 ± 89.6 g, range 58 – 410 g)

308   were regressed on telomere length at emergence. We further accounted for female mass at the

309   start of the breeding season as a covariate in the model for known influences of female starting

310   capital on reproduction (Broussard, Dobson, & Murie, 2005; Dobson, Risch, & Murie, 1999;

311   Rubach et al., 2016; Skibiel, Dobson, & Murie, 2009), as well as for assay-related terms (plate,

312   row within plate) as random variables. We initially controlled for female age as well, but

313   removed it from the final model since it explained virtually no variation and prevented models

314   from properly converging. In addition, we tested whether the increase in telomere length at the

315   start of the season (from emergence to birth) predicted litter size and mass at weaning (good

316   proxies of lactational investment; Skibiel, Dobson, & Murie, 2009). For this, we specified total

317   litter mass or total litter size at weaning as the dependent variables in separate models, and the

318   change in telomere length from emergence to parturition (hereafter birth) ($TL_{birth} - TL_{emergence}$)

319 as the independent variable of interest. Here also, we controlled for the influence of female

320 mass at the start of the breeding season by entering it as a covariate in the model.

321

322 *Telomere loss following reproduction*

323 We tested whether reproductive output was reflected in telomere loss. For this analysis, we

324 focused on female telomere dynamics during lactation, *i.e.* the period of highest energy

325 commitment to reproduction in mammals (Speakman, 2008), and regressed telomere change

326 from mid lactation to weaning ($TL_{weaning} - TL_{lactation}$, unscaled values) on litter mass and litter

327 size at weaning. Here also, we initially included assay-related terms (plate, row within plate)

328 and female age as random factors in the models. However, those variables explained virtually

329 no variation in telomere change, and we removed them from the final model as they prevented

330 models from properly converging. Then, we tested the correlation between female telomere

331 change from emergence to parturition ($TL_{birth} - TL_{emergence}$) and female telomere change from

332 mid lactation to weaning ($TL_{weaning} - TL_{lactation}$). This allowed us to determine if the magnitude

333 of maternal telomere change over lactation was related to the magnitude of maternal telomere

334 change at the start of the season, *i.e.* if females showing the greatest telomere loss during

335 lactation were also those that showed the highest amount of telomere reconstruction prior to

336 parturition.

337

338 *Statistical analyses*

339 All analyses were done in R v 4.0.2 (R Core Team 2021). We inspected model residuals for

340 normality using density distribution, Q-Q plots, cumulative distribution functions and P-P plots

341 obtained with the "fitdistrplus" package in R (Delignette-Muller & Dutang, 2015). Sample

342 sizes are indicated as the number of observations (n) and number of individuals (N).

343

RESULTS

Telomere length repeatability and relationship with female age and body mass

Controlling for assay-related terms (plate and row within plate), the repeatability of female relative telomere length over the season was low, but significant (r = 0.11; CI_{95} = [0.00, 0.26]; P = 0.044; Fig 1). Relative telomere length was significantly and negatively associated with female age (LMM; z-age estimate = -0.28, CI_{95} = [-0.51; -0.06], t = -2.51, P = 0.013), and positively associated with female mass (z-mass estimate = 0.27, CI_{95} = [0.08; 0.46], t = 2.79, P = 0.006).

Seasonal changes in telomere length

Controlling for female ID, age, mass and assay-related terms in our model, female relative telomere length varied in a quadratic fashion with advancing breeding season, and differed between females that did or did not raise a litter (LMM; period x status interaction; $F_{3,107.6}$ = 3.16, p = 0.028, n = 154 observations, N = 43 females; Fig 1, Table 1). For females that raised a litter, relative telomere length increased by 23%, though not quite significantly (Tukey HSD; t = -2.34, p = 0.096, Table 2), between emergence from hibernation to around the time of birth (i.e., during gestation). Relative telomere length then decreased significantly, by 28% between birth and weaning (Fig 1, Table 2). In females that did not raise a litter, relative telomere length also increased by 17% from emergence to what would correspond to around the time of birth for breeding females, and by another 10% to around the time of mid-lactation for breeding females (the increase from emergence to mid-lactation being significant; Fig 1, Table 2). In contrast to breeders, these females did not show a significant decrease in relative telomere length from the time of mid-lactation to the time of weaning of the offspring (Fig 1, Table 2). Interestingly, non-breeding females had significantly longer telomeres than breeding females

368   from around the time of mid-lactation (+20%; Tukey HSD; t = -2.1, p = 0.04) to the time when

369   breeding females weaned their offspring (+38%; Tukey HSD; t = -3.3, p = 0.001).

370

371   **Telomere length in relation to subsequent reproduction**

372   Controlling for maternal mass at emergence and assay-related terms, female telomere length at

373   emergence from hibernation neither significantly affected the size (LMM; z-telomere length =

374   0.08; CI_{95} = [-0.45; 0.60], t = 0.32, P = 0.753, N = 17 females) or mass (z-telomere length =

375   0.20; CI_{95} = [-36.73; 31.13], t = 0.01, P = 0.991, N=17 females) of the litter at weaning.

376   Similarly, controlling for maternal mass, telomere change at the start of the active season (from

377   emergence to birth; see Fig 1) did not significantly affect either litter size (z-telomere change

378   = 0.11; CI_{95} = [-0.65; 0.87], t = 0.32, P = 0.758, N = 16 females) or litter mass at weaning (z-

379   telomere change = 30.28; CI_{95} = [-17.47; 78.02], t = 1.41, P = 0.188, N = 16)

380

381   **Telomere loss following reproduction**

382   For females that raised a litter, telomere loss over lactation was significantly and negatively

383   related to both litter size (LM; z-litter size = -0.28; CI_{95} = [-0.53; -0.03], t = -2.38, P = 0.031;

384   R^2 = 0.23; N = 17; Fig 2A) and litter mass at weaning (z-litter mass = -0.32; CI_{95} = [-0.57; -

385   0.08], t = -2.83, P = 0.013; R^2 = 0.31; N = 17; Fig 2B). Female telomere changes at the start

386   and end of the breeding season were positively, but not significantly, correlated (Pearson's r =

387   0.39; CI_{95} = [-0.15; 0.75], t = 1.53, P = 0.149).

388

389   **DISCUSSION**

390   In Columbian ground squirrels, we found that telomere lengthening occurred between

391   emergence of hibernation through to about the time of births, some 26 days later, both for

392   breeding and non-breeding females (see Fig 1A). Afterwards, only breeding females

393   experienced significant shortening of their telomeres during the reproductive season, the

394   amplitude of telomere erosion being negatively related to litter size and mass (Fig. 2).

395   It has been suggested that telomere lengthening at emergence from hibernation might

396   occur to counteract the deleterious effects of hibernation (Hoelzl et al., 2016; Ruf & Bieber,

397   2020). The physiological costs of hibernation extend from impaired immune function (Cooper

398   et al., 1992) to enhanced oxidative stress, the latter being particularly problematic during

399   euthermic arousals associated with metabolic boosts and high production of reactive oxygen

400   species (ROS) (Orr et al., 2009). Telomeres may be a target of ROS (von Zglinicki 2002,

401   Reichert and Stier 2017; but see Boonekamp et al. 2017) and particularly vulnerable to

402   rewarming processes during euthermic arousals, due to sudden increases in oxidative

403   respiration (Murín et al., 2001). For instance, telomere shortening rates in hibernating edible

404   or garden dormice were positively related to the time spent euthermic during the inactive period

405   (Giroud et al., 2014), and to the frequency of arousals (Hoelzl et al., 2016). Reconstructing

406   chromosome ends at the start of the active period would be of particular importance if long

407   telomeres (or reduced annual shortening) are a seasonal prerequisite for individual survival

408   (Bize et al., 2009; Wood & Young, 2019). Though this must still be determined in Columbian

409   ground squirrels, associations between telomere length and survival have been documented

410   across vertebrates (Haussmann et al., 2003; Tricola et al., 2018; Vera et al., 2012; Whittemore

411   et al., 2019). Telomere maintenance (and rebuilding) may have coevolved with the relatively

412   slow pace of life that is characteristic of some hibernating rodents (*e.g.*, reduced mortality due

413   to winter inactivity; Turbill et al. 2011, Constant et al. 2020). In our study, although most

414   squirrels were captured on the day of emergence from hibernation (as known from the daily

415   survey of the study site and by the condition of the animal upon capture), we cannot exclude

416   the possibility that a few animals were caught slightly after the day of emergence. Thus, if

417   anything, telomere reconstruction following hibernation may have been slightly

418   underestimated in the present study, and may be even more pronounced than our current data

419   suggest.

420   In addition to counteracting the potentially deleterious effects of hibernation, findings

421   of telomere lengthening at the end of hibernation and onset of the active season (Hoelzl et al.,

422   2016; Ruf & Bieber, 2020; Turbill et al., 2013) question the importance of this process in

423   determining reproductive success and individual fitness in the wild. In this regards, telomere

424   elongation might be critical in at least two aspects: (*i*) by favoring reproduction, since greater

425   telomere length (or reduced telomere erosion) has been associated with greater reproductive

426   success (Angelier et al., 2019); or (ii) by serving as a pre-emptive change that mitigates the

427   physiological costs of reproduction (*i.e.*, shortened telomeres or higher telomere loss due to

428   reproduction; Bauch et al. 2012, Sudyka et al. 2014, Bichet et al. 2020, but see Sudyka 2019).

429   In our study, we found no clear relationship between telomere lengthening at the start

430   of the reproductive season (between emergence and birth) and female reproductive effort

431   measured as the litter size or mass produced at weaning. These results suggest that telomere

432   length at emergence of hibernation does not constitute a physiological constraint on

433   reproduction for females in this species. This is perhaps not surprising, given that female

434   Columbian ground squirrels are primarily income breeders: current reproductive effort depends

435   more strongly on current environmental conditions (Dobson & Oli, 2001), than on carried over

436   reserves and somatic condition from the previous year (Broussard et al., 2005; Rubach et al.,

437   2016). In contrast, female telomere length of somatic cells decreased from lactation to weaning

438   in breeding – but not in non-breeding (same relative chronological dates, see Methods) –

439   individuals. In addition, in breeding females, telomere length decreased in relation to

440   reproductive output: females producing larger and heavier litters experienced a higher telomere

441   loss (see Figs 1 and 2).

442   Telomere loss may be the ultimate cellular consequence of increased oxidative

443   metabolism and its inevitable production of reactive oxygen species ROS due to reproduction

444   (Kirkwood and Kowald 2012; but see Speakman et al. 2015). Several studies have found faster

445   telomere deterioration in breeding adults (Kotrschal et al. 2007, Reichert et al. 2014, Sudyka

446   et al. 2014, 2019, Bichet et al. 2020), even if the link between oxidative stress and telomere

447   loss remains unclear (Boonekamp et al., 2017). The present results seem to support the

448   hypothesis that reproduction entails physiological costs such as increased telomere loss (but

449   see Sudyka et al. 2019 for counter-examples). This is evidenced both by the difference between

450   breeding and non-breeding females, and by the positive association between telomere loss and

451   the increasing reproductive output by breeding females (*i.e.* litter size or mass).

452   Although ROS production during reproduction may contribute to shorter telomeres,

453   other mechanisms, such as different changes in hormonal levels in breeding and non-breeding

454   females across the reproductive cycle, might explain differential changes in telomere length.

455   For instance, elevated testosterone has been found to increase telomere loss (Heidinger et al.

456   2021), whereas increased progesterone has been found to increase telomerase activation (Kyo

457   et al., 1999). Both testosterone and oestrogen are likely to be higher in breeding females which

458   are territorial during lactation (Murie and Harris 1988), and their effects on telomere dynamics

459   (together with those of other hormones such as progesterone or prolactin) remain to be

460   investigated.

461   Of interest is the result that telomere elongation prior to reproduction may perhaps play

462   a role in mitigating the physiological costs of reproduction by pre-emptively reconstructing

463   telomeres before the costly period of lactation, as we previously documented with regards to

464   oxidative stress in this species (increased oxidative defences and reduced oxidative damage

465   during lactation; Viblanc et al. 2018). We indeed found a positive correlation ($r = 0.39$),

466   between pre-reproductive telomere elongation and post-reproductive telomere loss. However,

467   it important to note that the correlation was not significant, and additional data are therefore

468   needed to conclude if the reproductive physiology of females is geared towards mitigating all

469   of the physiological costs of lactation. One interesting question is how efficient this system

470   actually is: previous studies have failed to evidence long-term fitness costs to reproduction in

471   this species (Murie and Dobson 1987, Neuhaus 2000, Rubach et al. 2016; but see Neuhaus and

472   Pelletier 2001). Variability in individual ability to compensate for somatic and telomere

473   damage accumulated over hibernation also suggests that females of better individual quality

474   may better buffer the negative effects of high reproductive investments (Angelier et al., 2019;

475   Bauch et al., 2012; Sudyka, 2019). In line with this suggestion, we found a positive relationship

476   between female emergence mass and telomere length. Such an observation is consistent with

477   the idea that individual telomere length over the active period in female ground squirrels is

478   partly explained by differences in individual condition or quality (Sudyka, 2019). More work

479   is needed to clearly establish how far the consequences of reproduction on telomere length

480   result from a trade-off between actual costs on one hand (telomere attrition due to reproduction)

481   and variability in individual capacity to buffer these costs on the other.

482   Our findings add to the growing evidence that telomere length does not always reflect

483   unidirectional cellular ageing processes in the organism, including in adult animals. We found

484   that telomere dynamics in breeding females followed a seasonal pattern of reconstruction – and

485   shortening as a cost of reproduction. Our results suggest (*i*) that telomere reconstruction occurs

486   following hibernation in female Columbian ground squirrels, as in other hibernating mammals

487   (*ii*) that telomere loss over during the breeding season at least partly reflects a physiological

488   cost to reproduction, but (*iii*) we did not detect any effect of post-hibernating telomere

489   elongation on future reproductive success. Disentangling the importance of the (likely co-

490   occurring) processes of reproductive costs and variation in individual quality in buffering these

491   costs will require longitudinal surveys of life-long individual telomere dynamics and

492   reproductive success in animals, indicating the necessity of long-term studies in the wild.

493

507 **FIGURE CAPTIONS**

508 **Fig 1.** Relative telomere length dynamics in female Columbian ground squirrels over the course

509 of the breeding season. Results are marginal means ± 95% confidence intervals. Differences

510 between means were tested for relevant contrasts using Tukey HSD. Longitudinal differences

511 are indicated Table 2. Cross-sectional differences between groups at specific time points are

512 indicated by asterisks (**$P < 0.01$). Note that time intervals among sampling events are not

513 identical (see Methods). Sample sizes are given below the means for breeding (black) and non-

514 breeding (grey) categories. Relative telomere length was standardized (see Methods).

515

516 **Fig 2.** Relationship between relative telomere change over lactation and reproductive output in

517 female Columbian ground squirrels, measured as (a) litter size and (b) total litter mass (g) at

518 weaning (both variables standardized). The predictions and 95% confidence interval are given.

519 Relative telomere length was standardized (see Methods).

520

521

522

523 **TABLES**

524 **Table 1.** Mixed model estimates for the effects of breeding status (breeding [B] vs. non-breeding [NB]), period
525 in the breeding season (emergence from hibernation, shortly before births, at mid-lactation and at weaning – see
526 Methods) and their interaction on female relative telomere length in female Columbian ground squirrels.
527 Individual ID, age, mass, and assay-related terms (plate, row within plate) were included as random factors in the
528 models. Relative telomere length and independent variables are standardized (z-scores). N refers to individual and
529 n to TL sample size (repeated measurements on individuals).
530

Predictors	Estimates	CI	t	p	n(N)	R^2_{mar}/R^2_{cond}
(Intercept)	-0.50	-1.12 – 0.11	-1.62	0.108		
Period[Birth]	0.67	0.12 – 1.22	2.39	0.018*		
Period[Lactation]	0.33	-0.24 – 0.91	1.14	0.257		
Period[Weaning]	-0.36	-0.91 – 0.19	-1.28	0.201		
Status[NB]	0.11	-0.48 – 0.69	0.36	0.721		
Period[Birth] x Status [NB]	-0.15	-0.90 – 0.60	-0.40	0.693	154(43)	0.15 / 0.49
Period[Lactation] x Status [NB]	0.52	-0.25 – 1.30	1.34	0.183		
Period[Weaning] x Status [NB]	0.87	0.11 – 1.63	2.27	0.025*		

531
532
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534

535 **Table 2.** Tukey Honest Significant Differences for longitudinal mean comparisons in relative telomere length
536 (standardized, z-scores) for female Columbian ground squirrels at different time points in the breeding season.
537 Significant differences for P < 0.05 are indicated by asterisks.
538

| Status	Difference	Estimate ± SE	t ratio	P
Breeders	Emergence - Birth	-0.64 ± 0.27	-2.34	0.096
	Emergence - Lactation	-0.31 ± 0.28	-1.10	0.689
	Emergence - Weaning	0.34 ± 0.27	1.25	0.596
	Birth - Lactation	0.32 ± 0.27	1.20	0.629
	Birth - Weaning	0.98 ± 0.26	3.80	0.002*
	Lactation - Weaning	0.65 ± 0.27	2.46	0.073
Non-breeders	Emergence - Birth	-0.49 ± 0.25	-1.97	0.205
	Emergence - Lactation	-0.81 ± 0.25	-3.17	0.011*
	Emergence - Weaning	-0.48 ± 0.26	-1.87	0.246
	Birth - Lactation	-0.32 ± 0.25	-1.27	0.585
	Birth - Weaning	0.01 ± 0.25	0.05	1.000
	Lactation - Weaning	0.33 ± 0.26	1.29	0.571

539

540 **FIGURES**
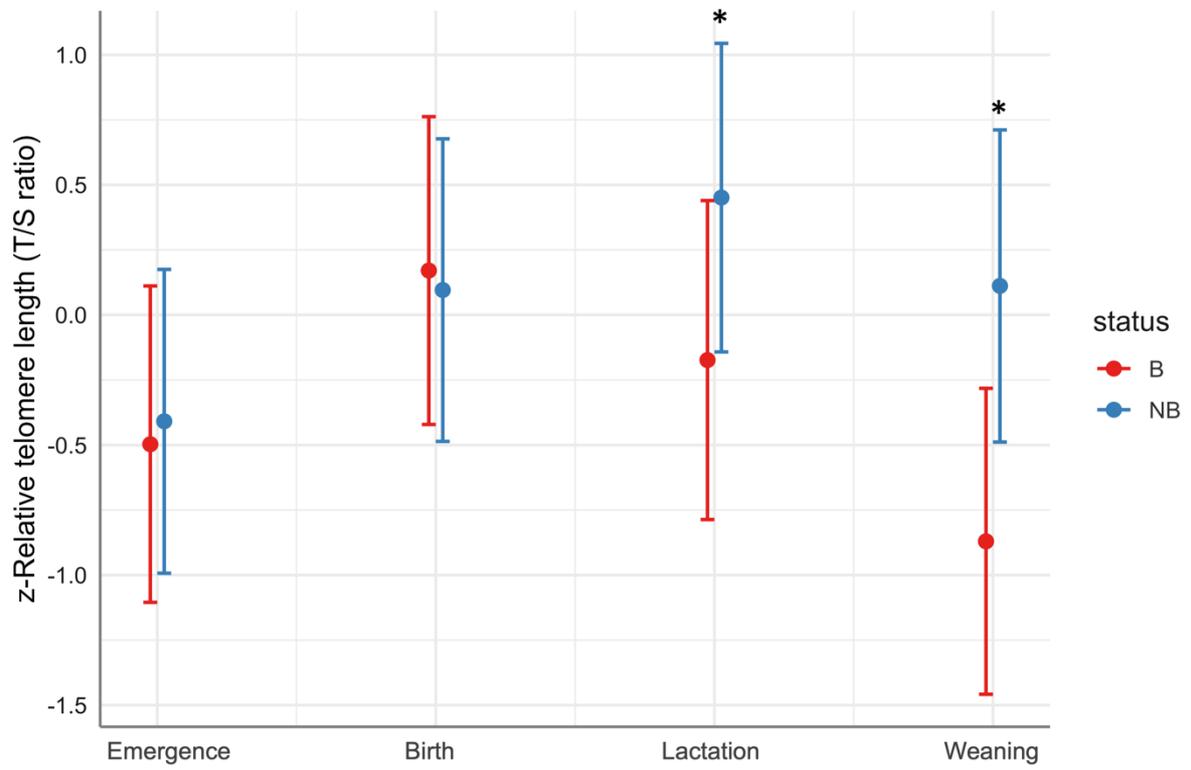
541

542

543 **Fig 1.**

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547
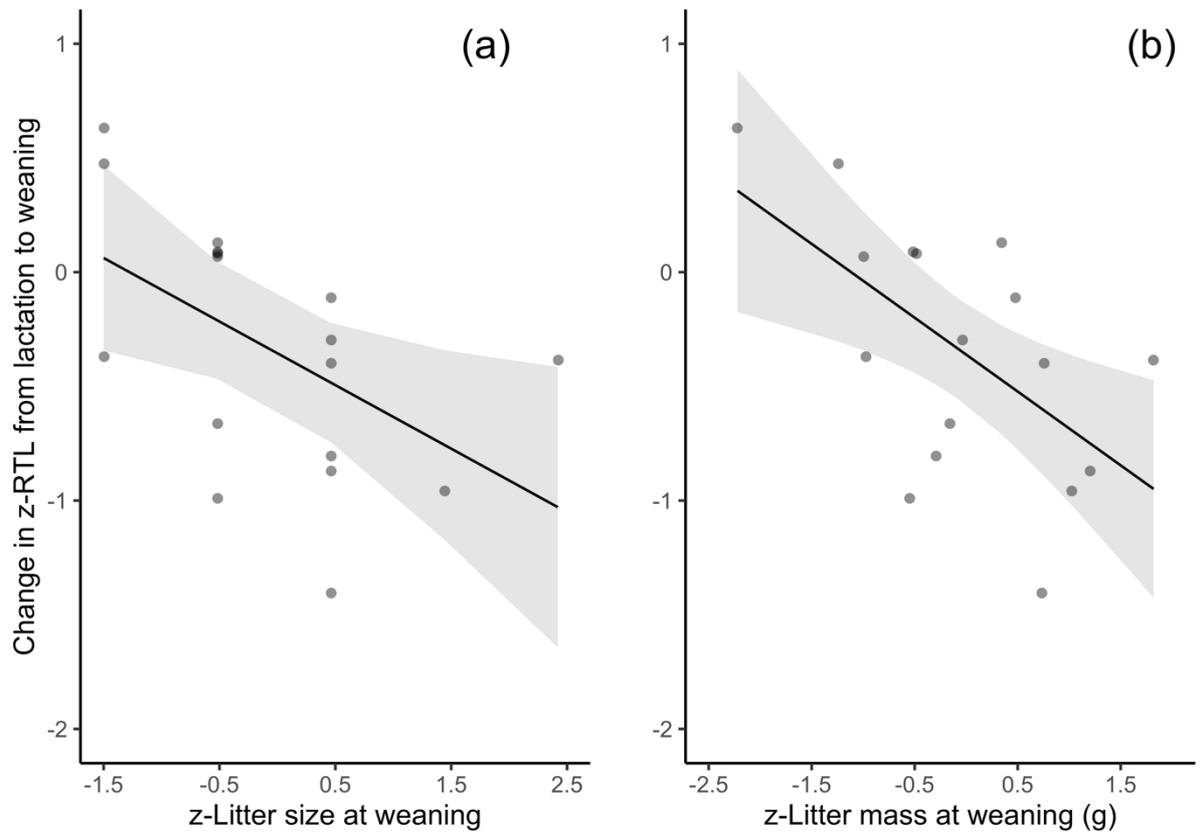
Fig 2.

549

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DECLARATIONS

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