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1 **Telomere dynamics in hibernating female Columbian ground**
2 **squirrels: recovery after emergence and loss after reproduction**

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15 **Keywords**

16 Aging, telomere, social stress, reproduction, hibernation, mammals

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

25 **ABSTRACT**

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

44

45 **Keywords:** telomeres, cost of reproduction, reproductive constraint, hibernation, telomerase

46 INTRODUCTION

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

60 Beside the end-replication problem, a growing number of studies have highlighted how
61 telomere length may be an integrative proxy of individual stress, as telomere length integrates
62 the accumulation of stress-related damages with age, and thus reflects individual life
63 experiences (Dupoué et al., 2020; Monaghan & Haussmann, 2006). For instance,
64 environmental harshness (*e.g.* pollution levels, disease prevalence, oxidative stress,
65 environmental and social stressors; reviewed by Chatelain, Drobniak, & Szulkin, 2020) has
66 been shown to negatively affect telomere length in groups as diverse as fish (Molbert et al.,
67 2021), birds (Asghar et al., 2015; Aydinonat et al., 2014; Grunst et al., 2020), and mammals
68 (Kesäniemi et al., 2019), including humans (Blackburn & Epel, 2012). As data grow, so does
69 our understanding of the importance of these highly conserved DNA structures in shaping life-
70 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 (*Uroditellus 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 (Skibieli, 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 (Viblanco 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

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

152

153 **METHODS**

154 **Study site and population monitoring**

155 Female Columbian ground squirrels were monitored in a 2.6 ha subalpine meadow from 1992
156 to 2019 in the Sheep River Provincial Park, Alberta, Canada (50° 38' 10.73" N; 114° 39'
157 56.52" W; 1520 m), as part of a long-term study on their ecology, behavior, and physiology
158 (Dobson et al. 2020). Reproduction takes place over the short summer season (3-4 months),
159 during which sexually mature females (2-14 years old) produce a single litter (2-7 pups) over
160 51 days (24 days of gestation and *ca* 27 days of lactation and post-weaning parental care, Murie
161 and Harris 1982). In each year, the entire population was trapped at emergence from
162 hibernation, using 13 x 13 x 40 cm traps (Tomahawk Live Traps; Hazelhurst, WI, USA) baited
163 with a small amount of peanut butter. Upon trapping, each ground squirrel was weighed to the
164 nearest 5 g (Pesola spring-slide scale; Pesola® Ag; Baar, Switzerland), tagged if unmarked
165 (#1-Monel metal tag; National Band and Tag Company, Newport, KY) and painted with a
166 unique dorsal mark for visual identification with black hair dye (Clairol, Stamford, CT).
167 Consequently, all females in the population were of known age. Buccal tissue for telomere
168 length (see below) was collected during the 2019 active period.

169 We observed animals daily (from ~ 7:00 am to 2:00 pm), starting at emergence from
170 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 μ L 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 μ L reaction. Each sample of DNA was diluted to 2.5 ng/ μ L with double-distilled
227 H₂O just prior to running the reactions, and 2 μ L of this sample is used in each 10 μ L 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/ μ L).
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/ μ L to 0.3125 ng/ μ L) to evaluate
243 plate amplification efficiencies of the telomere sequence and the non-VCN gene, and to check
244 that the C_q 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:

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

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

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

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 (\pm 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; Skibieli, 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; Skibieli, 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) ($TL_{\text{birth}} - TL_{\text{emergence}}$)

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 ($TL_{\text{weaning}} - TL_{\text{lactation}}$, 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 ($TL_{\text{birth}} - TL_{\text{emergence}}$) and female telomere change from
332 mid lactation to weaning ($TL_{\text{weaning}} - TL_{\text{lactation}}$). 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

344 **RESULTS**

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

346 Controlling for assay-related terms (plate and row within plate), the repeatability of female
347 relative telomere length over the season was low, but significant ($r = 0.11$; $CI_{95} = [0.00, 0.26]$;
348 $P = 0.044$; Fig 1). Relative telomere length was significantly and negatively associated with
349 female age (LMM; z-age estimate = -0.28 , $CI_{95} = [-0.51; -0.06]$, $t = -2.51$, $P = 0.013$), and
350 positively associated with female mass (z-mass estimate = 0.27 , $CI_{95} = [0.08; 0.46]$, $t = 2.79$, P
351 = 0.006).

352

353 **Seasonal changes in telomere length**

354 Controlling for female ID, age, mass and assay-related terms in our model, female relative
355 telomere length varied in a quadratic fashion with advancing breeding season, and differed
356 between females that did or did not raise a litter (LMM; period x status interaction; $F_{3,107.6} =$
357 3.16 , $p = 0.028$, $n = 154$ observations, $N = 43$ females; Fig 1, Table 1). For females that raised
358 a litter, relative telomere length increased by 23%, though not quite significantly (Tukey HSD;
359 $t = -2.34$, $p = 0.096$, Table 2), between emergence from hibernation to around the time of birth
360 (i.e., during gestation). Relative telomere length then decreased significantly, by 28% between
361 birth and weaning (Fig 1, Table 2). In females that did not raise a litter, relative telomere length
362 also increased by 17% from emergence to what would correspond to around the time of birth
363 for breeding females, and by another 10% to around the time of mid-lactation for breeding
364 females (the increase from emergence to mid-lactation being significant; Fig 1, Table 2). In
365 contrast to breeders, these females did not show a significant decrease in relative telomere
366 length from the time of mid-lactation to the time of weaning of the offspring (Fig 1, Table 2).
367 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 (Hausmann 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

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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 \pm 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**

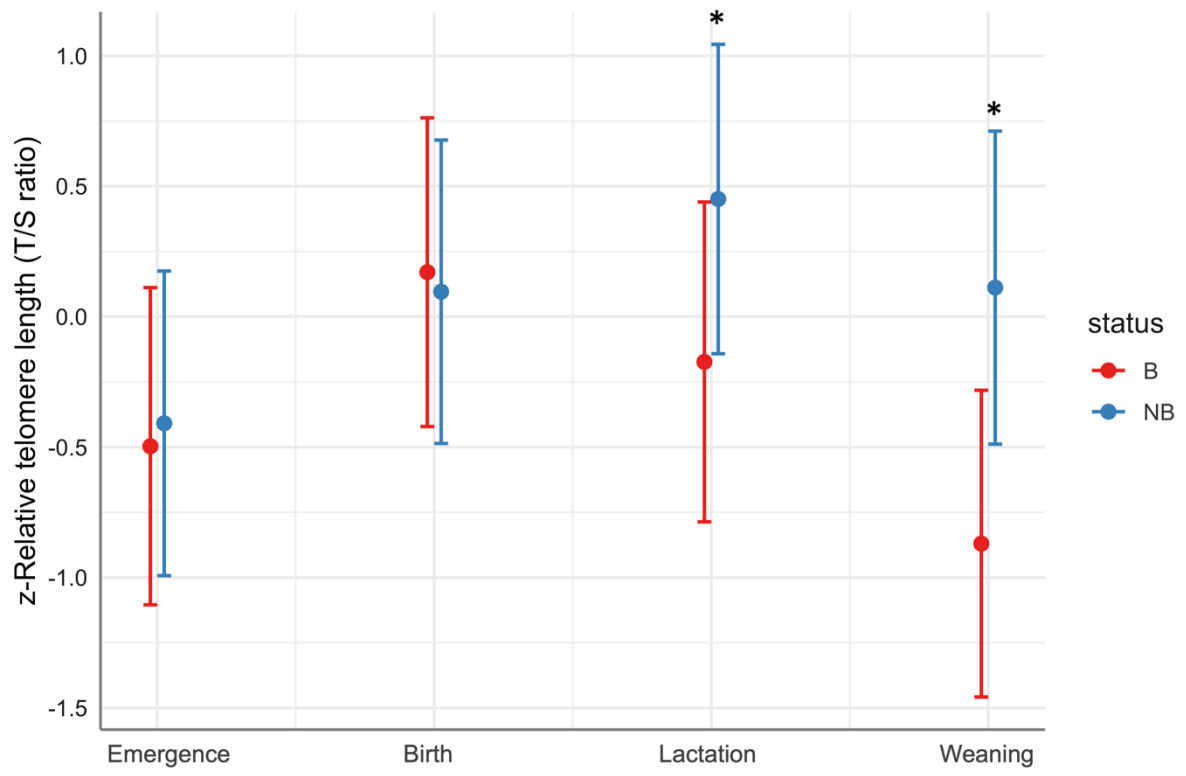
524 **Table 1.** Mixed model estimates for the effects of breeding status (breeding [B] vs. non-breeding [NB]), period
 525 in the breeding season (emergence from hibernation, shortly before births, at mid-lactation and at weaning – see
 526 Methods) and their interaction on female relative telomere 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

<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>t</i>	<i>p</i>	<i>n(N)</i>	<i>R²_{mar}/R²_{cond}</i>
(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
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 533
 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

<i>Status</i>	<i>Difference</i>	<i>Estimate ± SE</i>	<i>t ratio</i>	<i>P</i>
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



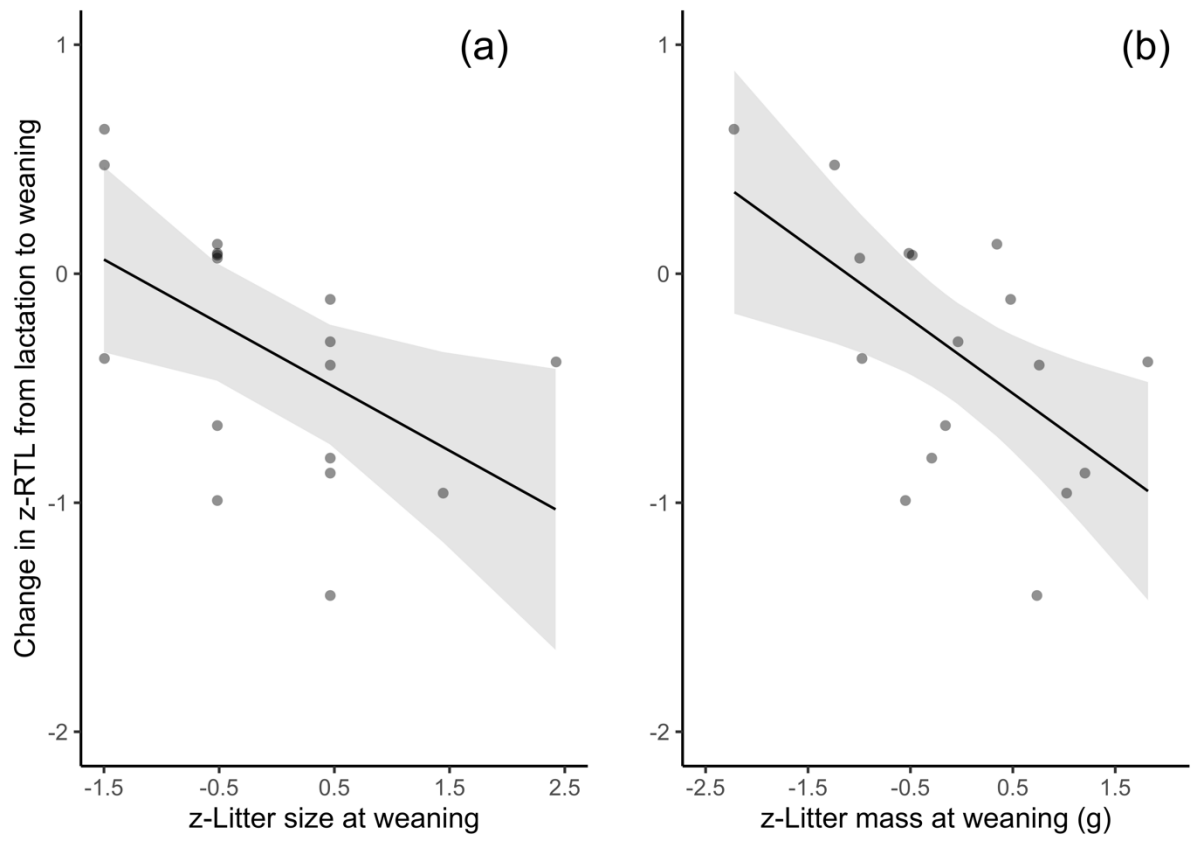
541

542

543 **Fig 1.**

544

545



547

548 **Fig 2.**

549

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