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

Vincent A. Viblanc, Francois Criscuolo, Sebastian Sosa, Quentin Schull, Rudy Boonstra, Claire Saraux, Mathilde Lejeune, Jeffrey D. Roth, Pierre Uhrich, Sandrine Zahn, et al.

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

3  
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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 =  $4.4 \pm 1.9$  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 =  $2.6 \pm 2.0$  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  $\mu$ 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  $\mu$ L reaction. Each sample of DNA was diluted to 2.5 ng/ $\mu$ L with double-distilled  
227 H<sub>2</sub>O just prior to running the reactions, and 2  $\mu$ L of this sample is used in each 10  $\mu$ 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/ $\mu$ 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/ $\mu$ L to 0.3125 ng/ $\mu$ L) to evaluate  
243 plate amplification efficiencies of the telomere sequence and the non-VCN gene, and to check  
244 that the C<sub>q</sub> 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) =  $2.5 \pm 1.0$ ,  
307 range 1 – 5) or total litter mass (N = 18, total litter mass =  $247.2 \pm 89.6$  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

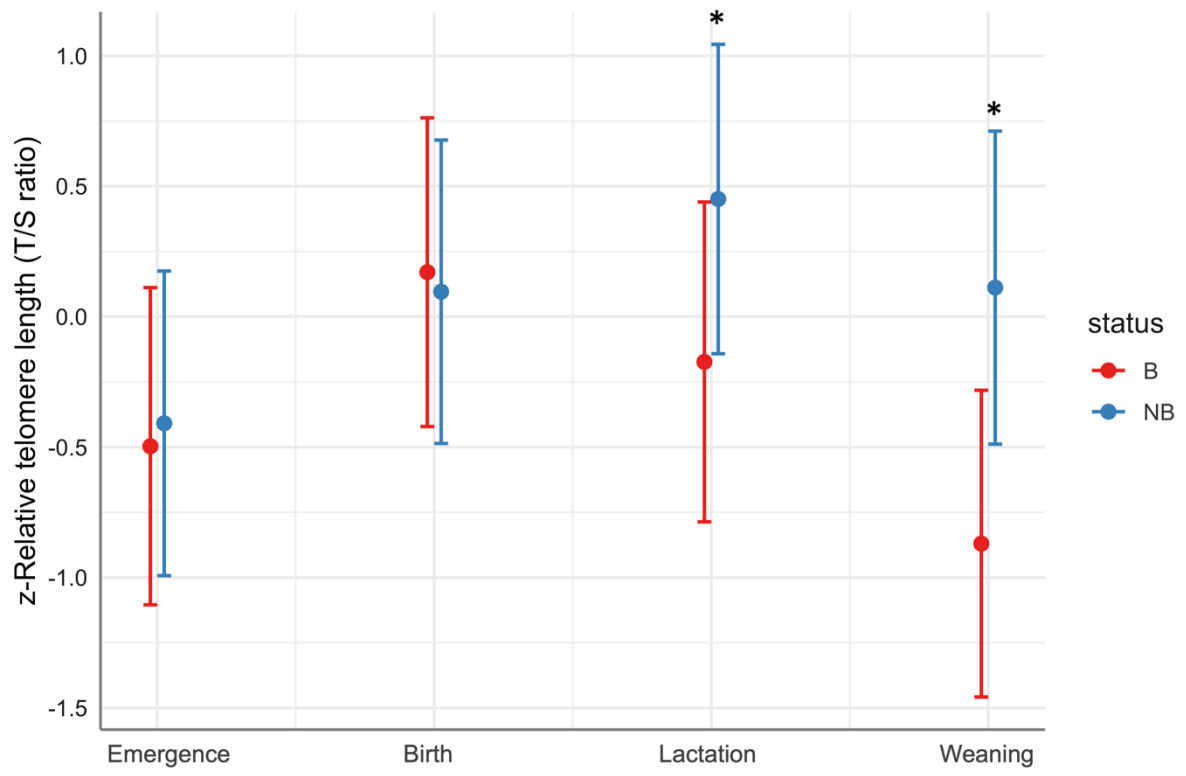
<i>Predictors</i>	<i>Estimates</i>	<i>CI</i>	<i>t</i>	<i>p</i>	<i>n(N)</i>	<i>R<sup>2</sup><sub>mar</sub>/R<sup>2</sup><sub>cond</sub></i>
(Intercept)	-0.50	-1.12 – 0.11	-1.62	0.108		
Period <sub>[Birth]</sub>	0.67	0.12 – 1.22	2.39	0.018*		
Period <sub>[Lactation]</sub>	0.33	-0.24 – 0.91	1.14	0.257	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

531  
 532  
 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



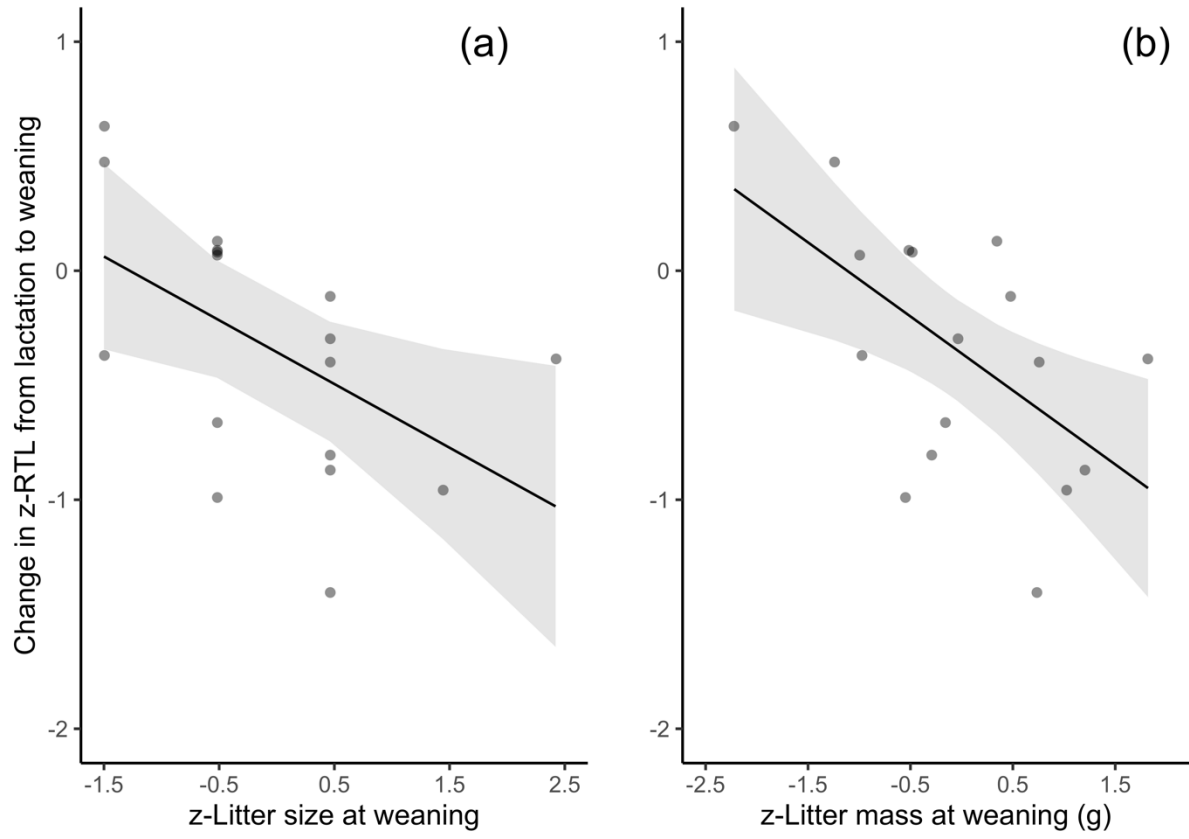
541

542

543 **Fig 1.**

544

545



547

548 **Fig 2.**

549

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