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

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Author contribution statement
VAV, FSD and FC designed the study. VAV, SS, CS, JFR, QS and FSD collected the data in the field. VAV and CS performed the statistical analysis and ML and SZ the laboratory analyses and check the quality of the qPCR results with FC. VAV and FC wrote the first draft of the manuscript. ML and SZ wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.
Telomeres are specialized non-coding DNA sequences at the end of chromosomes that protect genetic information. Telomere loss over lifespan is generally viewed as a phenomenon associated with ageing in animals. Recently, telomere elongation after hibernation has been described in several mammals. Whether this pattern is an adaptation to repair DNA damage caused during rewarming from torpor or if it coevolved as a mechanism to promote somatic maintenance in preparation for the upcoming reproductive effort remains unclear. In a longitudinal study measuring telomere length using buccal swabs, we tested if telomere elongation was related to reproductive success in wild adult female Columbian ground squirrels \textit{(Urocitellus columbianus)} that were monitored from emergence from hibernation to the end of the reproductive season. We found three key results. First, female telomere length increased at the start of the breeding season, both in breeding and non-breeding individuals. Second, post-emergence telomere lengthening was unrelated to female future reproductive output. Third, telomere length decreased in breeding females during lactation, but remained stable in non-breeding females over a similar period. Within breeders, telomeres shortened more in females producing larger and heavier litters. We concluded that telomere lengthening after hibernation did not constrain immediate female reproductive capacities. It was more likely to be part of the body recovery process that takes place after hibernation. Telomere erosion that occurs after birth may constitute a physiological cost of female reproduction.

\textbf{Keywords:} telomeres, cost of reproduction, reproductive constraint, hibernation, telomerase
INTRODUCTION

Telomeres are sequences of non-coding DNA that cap the end of linear chromosomes and protect the integrity of coding DNA where key genetic information resides (Greider, 1991).

As cells replicate, telomeres progressively shorten due to the end-replication problem of DNA (viz., the extremities of DNA on the lagging-strand are lost due to a lack of template) (Harley, Futcher, & Greider, 1990; Watson, 1972). Although part of this replication problem is counterbalanced by telomerase, an enzyme that rebuilds otherwise dwindling telomeres (Greider & Blackburn, 1985), critically short telomeres lead to cells leaving the normal replicative cycle through cell apoptosis (Blackburn, 1991). From an organismal perspective, telomere length, and maybe more so the rate of telomere loss, are increasingly considered reliable proxies of individual or species lifespan, both in captive (e.g. Heidinger et al., 2012; Whittemore et al., 2019) and free-living vertebrates (reviewed by Wilbourn et al. 2018).

Telomeres are associated with individual fitness in numerous species (Bichet et al., 2020; Dupoué et al., 2017; van Lieshout et al., 2019).

Beside the end-replication problem, a growing number of studies have highlighted how telomere length may be an integrative proxy of individual stress, as telomere length integrates the accumulation of stress-related damages with age, and thus reflects individual life experiences (Dupoué et al., 2020; Monaghan & Haussmann, 2006). For instance, environmental harshness (e.g. pollution levels, disease prevalence, oxidative stress, environmental and social stressors; reviewed by Chatelain, Drobiak, & Szulkin, 2020) has been shown to negatively affect telomere length in groups as diverse as fish (Molbert et al., 2021), birds (Asghar et al., 2015; Aydininat et al., 2014; Grunst et al., 2020), and mammals (Kesäniemi et al., 2019), including humans (Blackburn & Epel, 2012). As data grow, so does our understanding of the importance of these highly conserved DNA structures in shaping life-history trade-offs and tactics across species (Dantzer & Fletcher, 2015; Young, 2018).
Telomere characteristics are likely to shed useful mechanistic insights on life histories. This is because telomere loss may reflect the physiological costs that an individual has to pay when limited energy is invested in potentially competing, resource-demanding life-history functions (such as reproduction and self-maintenance). This is based on the hypothesis that telomere maintenance is an energetically costly mechanism (Young, 2018). Whereas we have no direct evidence for this hypothesis so far, indirect evidence has been accumulating from studies focusing on reproductive costs and from hibernating species (e.g. Hoelzl et al., 2016; Nowack et al., 2019).

In fact, experimental increases in long-term reproductive effort and actuarial senescence (increased parental mortality rates) have been positively correlated (Boonekamp, Bauch, & Verhulst, 2020), suggesting that reproduction somehow triggers ageing mechanisms in adults (Flatt & Heyland, 2011). Experimentally increasing reproductive effort was found to hasten telomere shortening of breeding birds (Reichert et al., 2014; Sudyka et al., 2014; for contrasting results see Sudyka et al., 2019). While most experimental studies published to date supported the hypothesis of accelerated telomere erosion as a cost of reproduction, negative associations between reproductive traits and telomere dynamics were only found in roughly half of the studies conducted in wild vertebrates (Sudyka et al. 2019). Thus, whether the negative impact of reproduction on telomeres is a general phenomenon across vertebrates remains to be determined (e.g. Sudyka et al. 2016; Sudyka et al., 2019), and further evidence is needed, particularly in non-avian species.

Previous studies on hibernating rodents convincingly demonstrate that telomere maintenance is achieved at energetic costs. Hibernation is composed of periods of decreased body temperature (torpor) and rewarming events, and the frequency of these torpor events or rewarming events and associated metabolic activation have been related to telomere dynamics (Turbill et al., 2012; Turbill et al., 2013; Hoelzl et al. 2016). Interestingly, experimental
manipulation of food supply or of ambiant temperature during or after hibernation were shown
to impact both telomere shortening or elongation, suggesting an energy cost of telomere
maintenance (Hoelzl et al. 2016, Nowack et al. 2019). In any case, hibernating species appear
to retain the seasonal ability to reconstruct their telomeres after hibernation (i.e. at the start of
the active season, Hoelzl et al., 2016; Turbill et al., 2012; Turbill et al., 2013). Such a
phenomenon may characterize the switch of adult physiology from a non-active hibernating
state to an active reproductive state (Hoelzl et al., 2016), and has been suggested as an
immediate post-hibernating and pre-reproductive strategy (Nowack et al., 2019), but its actual
benefits in terms of fitness remain unclear. Telomere elongation at emergence from hibernation
could occur as part of a somatic maintenance process related to hibernation (Turbill, Bieber, &
Ruf, 2011), as an anticipatory process in order to buffer the oxidative (telomere-shortening)
costs of upcoming reproduction (i.e. oxidative shielding hypothesis; Blount et al. 2016, Viblanc
et al. 2018), or both. Teasing apart those alternatives requires information from hibernating
species in which seasonal telomere dynamics at the individual level are assessed at emergence
from hibernation and then longitudinally throughout the breeding season. By comparing
breeding and non-breeding individuals sampled at similar time periods, it is possible to test
whether telomere length, telomere lengthening or telomere loss predict individual reproductive
success, or reflect a cost of reproduction, while controlling for individual differences in age
and condition (i.e., inter-individual differences in body mass at the onset of the breeding
season).

We tested the hypothesis that telomere dynamics (both lengthening and shortening)
varied as a response to hibernation and reproduction in free-living female Columbian ground
squirrel (Urocitellus columbianus). Columbian ground squirrels provide a robust model system
for addressing these questions. These montane rodents have an especially short active season
(3-4 months) during which females emerge, soon mate, gestate (ca. 24 days), lactate and raise
their young (ca. 27 days), molt and fatten before subsequent hibernation (Dobson, Badry, & Geddes, 1992; Dobson & Murie, 1987). The reproductive season starts shortly after emergence from hibernation, and females typically enter oestrus 3-4 days following emergence (Lane et al. 2011). Thus, this is a short and highly active period following a long period of metabolic inactivity. For females, lactation is an energy-demanding period when oxidative metabolism is high (Skibiel, Speakman, & Hood, 2013; Speakman, 2008) and when the oxidative costs of reproduction are expected to be highest. Interestingly, these females seem to have evolved increased antioxidant defenses, which are highest during lactation (Viblanc et al. 2018), allowing them to buffer the oxidative costs of reproduction (i.e. the oxidative shielding hypothesis; Blount et al. 2016). This suggest that lactation entails physiological costs, but whether those costs extend to damage to telomeres remains to be tested.

We hypothesized that, at the start of the breeding season (from emergence of hibernation to parturition), an increase in telomere length might be expected both with regards to somatic maintenance/reconstruction following hibernation and as part of a mechanistic process that buffers the later oxidative costs of lactation (Blount et al., 2016). On one hand, and after controlling for individual age and condition, a positive association between starting telomere length and reproductive output would suggest that telomere length acts as a constraint on reproduction (i.e., females with low somatic condition would not invest in reproduction). On the other, a positive relationship between reproductive output and telomere loss over lactation would suggest that reproductive effort indeed entails physiological costs (i.e., females with high reproductive outputs exhibit greater reduction in telomere length over the breeding season). To test this, we followed known-aged females at emergence from hibernation and over the course of the breeding season. Since nothing is known so far about how variable telomere length is in this species, or whether telomere length is related to individual age or body condition, we first evaluated the individual repeatability of telomere length over the breeding
season, and the potential effect of female age and condition (mass) on telomere length. Then, we evaluated: (i) if female telomere lengthening occurred at the start of the breeding season, from emergence of hibernation to parturition; (ii) if telomere length at emergence from hibernation (and potential subsequent reconstruction from emergence to parturition) predicted female reproductive output (assessed by litter size and mass measured at weaning); and (iii) if female reproductive output was associated with increased telomere loss during lactation.

METHODS

Study site and population monitoring

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

We observed animals daily (from ~ 7:00 am to 2:00 pm), starting at emergence from hibernation and continuing throughout the reproductive season. We determined mating dates
for each female from male-female interactions, the occurrence of below-ground consortships or above-ground mating events (Raveh et al., 2010, 2011). All females were surveyed daily until we were certain that they had (or had not) reproduced, as confirmed by mating behavior. When in doubt, we inspected female genitalia for the presence of copulatory plug material and dried sperm as indicators of successful mating events (Murie & Harris, 1982). We flagged the location of female nest-burrows (single-entrance burrows where females raise and lactate to feed their young), identified by daily observations of female’s morning emergences from these burrows and observations of females stocking them with loads of dry grass. Mothers and their entire litters were trapped at about the time of weaning, when offspring first emerged above ground, approximately 51 days after mating and 27 days after parturition. All offspring were weighed to the nearest 1 g and given unique ear tags and dye markings.

Tissue collection and telomere length measurement

Tissue collection

Cell tissue was collected from the buccal mucosa of each ground squirrel by gently twirling a Gynobrush® brush (Heinz Herenz Medizinalbedarf, Hamburg) on the inside of each cheek. This technique is particularly adapted for repeated non-invasive sampling (Hoelzl et al. 2016), and buccal cell DNA to reflect individual health status (e.g. in humans, Thomas et al. 2008)

The collected tissue was immediately transferred to 96% ethanol Eppendorf tubes and kept at 4°C until DNA extraction. For breeding females (N = 19; age = 4.4 ± 1.9 years old, range = 2 – 8 years old), tissue was collected at four points calculated for each individual: emergence from hibernation (or the day after), and then 26, 38 and 54 days later (around the times of parturition, mid-lactation, and offspring weaning). Tissue was collected from non-breeding females (non-breeding females: N = 24; age = 2.6 ± 2.0 years old, range = 1 – 7 years old) following a similar schedule. Overall, we were able to acquire tissue for 43 females including
19 breeding and 24 non-breeding individuals all sampled four times (i.e. 172 samples). However, differences in sample sizes between our different analyses are due to varying success at acquiring samples in the field or telomere amplification in the laboratory, or missing data in other parameters on some occasions (e.g., female mass).

**DNA extraction**

DNA extraction was carried out using the Nucleospin Tissue kit, Macherey-Nagel, Düren, Germany, and checked for quality using gel-migration (DNA integrity) and a NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA, USA) spectrophotometer (absorbance ratio A260/280; A260/230, DNA quality). Briefly, after allowing ethanol to evaporate from the tissue sample, lysis was achieved by incubation of the sample material in a proteinase K / SDS solution. Appropriate conditions for DNA binding to the silica membrane in the NucleoSpin® Tissue Columns were achieved by adding chaotropic salts and ethanol to the lysate. The binding process was reversible and specific to nucleic acids. Contaminants were removed by subsequent washing with two different buffers as indicated by the kit’s protocol. Pure genomic DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.

**Telomere qPCR amplification**

Extracted DNA was used to amplify both the telomere sequence and a control gene by quantitative real-time amplification (qPCR) based on Cawthon’s (2002) original publication. As a reference gene that was invariant in copy number (non-VCN, Smith et al. 2011), we used a 54 bp portion of the c-myc proto-oncogene, which was tested for non-variability in copy number in our population using amplicon gel migration (see Online Supplementary Materials). Forward and reverse telomeric primers were 5’- CGG TTT GTT TGG GTT TGG GTT TGG GTT GTT TGG GTT - 3’ and 5’- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC TAC TAC
Primer sequences for the non-VCN gene were 5’- GAG GGC CAA GTT GGA CAG TG - 3’, and 5’- TTG CGG TTG TTG CTG ATC TG -3’. A master mix was prepared for each primer set containing 5 µL GoTaq® qPCR Master Mix (Promega, Madison, WI, USA).

We used telomere primers at a concentration of 200 nM and non-VCN primers at 400 nM in a 10 µL reaction. Each sample of DNA was diluted to 2.5 ng/µL with double-distilled H₂O just prior to running the reactions, and 2 µL of this sample is used in each 10 µL reaction.

qPCR conditions for telomeres were 2 min at 95°C followed by 30 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C. qPCR conditions for the non-VCN gene were 2 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 59°C and 1 min at 72°C. Amplifications were done on a 384-well thermocycler (CFX-384 Touch Real-Time PCR Detection System, Biorad, USA). Duplicates of each sample’s telomere and non-VCN qPCR amplifications were performed on separate plates (i.e. forming a qPCR run), the amplification conditions being different between telomere and non-VCN sequences (see above). In total, all samples of our experiment were measured over 2 runs.

In addition to our ground squirrel samples, all plates included a no template control (water) and a ‘calibrator’ sample in duplicate. The calibrator was DNA extracted from a single individual (golden sample randomly chosen among those for which a large quantity of DNA was available), diluted to the same concentration as other ground squirrel samples (2.5 ng/µL). Both a negative control (water) and melting curves were run for each plate to control for the absence of (i) non-specific amplification and of (ii) primer-dimer artefact. On each plate, we also included a calibrator sample dilution curve (from 10 ng/µL to 0.3125 ng/µL) to evaluate plate amplification efficiencies of the telomere sequence and the non-VCN gene, and to check that the Cq values produced declined in a log-linear fashion ($r^2 > 0.98$) before proceeding to statistical analysis.
Among the amplification values of the control gene, we had 23 samples (13.4% of 172 samples,) which were delayed of 1 Cq compared to the mean Cq value. Running the analyses with or without these samples yielded similar results, and thus we chose to keep them in the present analysis. Efficiencies for the two reactions were 0.997 (range 0.996 – 0.999) for non-VCN genes and 0.996 (range 0.991- 1.001) for telomeres. We calculated relative telomere length (RTL) following Pfaffl (2001), as:

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

where \( E_{TEL} \) and \( E_{non-VCN} \) are the mean plate efficiencies for each sequences, and \( Cq_{TEL[calibrator]} \) and \( Cq_{TEL[sample]} \) are the mean Cq for telomere calibrator and sample, respectively. Similarly, \( Cq_{non-VCN[calibrator]} \) and \( Cq_{non-VCN[sample]} \) are the mean Cq for non-VCN calibrator and sample, respectively. The intraclass correlation coefficient (ICC) was calculated for intra-run and inter-run variation of T/S ratio following (Cicchetti 1994), and was recorded at 0.783 and 0.805, respectively.

**Data analyses**

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

First, we used a linear mixed model (LMM) approach to estimate individual repeatability in telomere length over the course of a breeding season as

\[ R = \frac{V_G}{V_P} = \frac{V_G}{V_G + V_R} ; \]

\( V_G \) is the among-individual variance in telomere length, \( V_P \) the total phenotypic variance in telomere length, and \( V_R \) the within-individual (or residual) variance in telomere length (Nakagawa and Schielzeth 2010, Stoffel et al. 2017). We accounted for potential error measurement associated with qPCR assays by including assay-related terms (plate, row within plate) as random factors in the model. Confidence intervals around repeatability estimates were
calculated by parametric bootstrapping (10,000 iterations) using the ‘rptR’ R cran package (Stoffel et al., 2017). The statistical significance of repeatability estimates was calculated using likelihood ratio tests, comparing the model fit to models where group-level variance was constrained to zero.

Second, we ran a LMM (‘lme4’ package in R) to test whether female telomere length was related to female age or condition (estimated as mass) at the time of measurement. For this, female telomere length was specified as the dependent variable, and female age and mass as independent variables. We included female ID as well as assay-related terms (plate, row within plate) as random factors in the model to account for repeated measures on individual females over the breeding season, and potential measurement error associated with qPCR assays. Telomere length and independent variables were systematically standardized (z-scores) prior to analyses to facilitate future comparisons between q-PCR-based telomere studies (Verhulst, 2020), and so that estimates of models for independent variables could be directly compared as effect sizes.

Seasonal changes in telomere length

Seasonal changes in telomere length were investigated separately in females using a LMM, with telomere length specified as the dependent variable, and seasonal timing (emergence vs. birth vs. lactation vs. weaning), breeding status (breeder vs. non-breeder), and their interaction as independent variables. Breeding females were those observed lactating and raising a litter. We specifically tested for the interaction between breeding status and seasonal timing, to test whether seasonal dynamics in telomere length were different between breeding and non-breeding females. Because our previous analyses showed significant influences of female age and mass on telomere length, we controlled for those variables, as well as for female ID (repeated measures over the season) and assay-related terms (plate, row within plate) as random factors in the model.
factors in the model. As above, all variables were standardized. As a measure of effect size, we
calculated percent changes in telomere length between seasonal time periods from marginal
means obtained from a similar LMM but where telomere length was not standardized (so that
actual percent differences between time periods could be calculated). Significant differences
between time periods were determined by Tukey Honest Significant Difference post-hoc tests
(HSD), which control for multiple testing, using the ‘emmeans’ package. Marginal model
means are presented along with their 95% confidence intervals in the figures and significant
differences along with their 95% confidence intervals are presented in the tables.

Telomere length in relation to subsequent reproduction

For those females that bred, we tested whether telomere length at the start of the breeding
season predicted female reproductive output. Litter size (N = 18, litter size (± SD) = 2.5 ± 1.0,
range 1 – 5) or total litter mass (N = 18, total litter mass = 247.2 ± 89.6 g, range 58 – 410 g)
were regressed on telomere length at emergence. We further accounted for female mass at the
start of the breeding season as a covariate in the model for known influences of female starting
capital on reproduction (Broussard, Dobson, & Murie, 2005; Dobson, Risch, & Murie, 1999;
Rubach et al., 2016; Skibiel, Dobson, & Murie, 2009), as well as for assay-related terms (plate,
row within plate) as random variables. We initially controlled for female age as well, but
removed it from the final model since it explained virtually no variation and prevented models
from properly converging. In addition, we tested whether the increase in telomere length at the
start of the season (from emergence to birth) predicted litter size and mass at weaning (good
proxies of lactational investment; Skibiel, Dobson, & Murie, 2009). For this, we specified total
litter mass or total litter size at weaning as the dependent variables in separate models, and the
change in telomere length from emergence to parturition (hereafter birth) (TL_{birth} – TL_{emergence})
as the independent variable of interest. Here also, we controlled for the influence of female mass at the start of the breeding season by entering it as a covariate in the model.

Telomere loss following reproduction

We tested whether reproductive output was reflected in telomere loss. For this analysis, we focused on female telomere dynamics during lactation, i.e. the period of highest energy commitment to reproduction in mammals (Speakman, 2008), and regressed telomere change from mid lactation to weaning ($TL_{\text{weaning}} - TL_{\text{lactation}}$, unscaled values) on litter mass and litter size at weaning. Here also, we initially included assay-related terms (plate, row within plate) and female age as random factors in the models. However, those variables explained virtually no variation in telomere change, and we removed them from the final model as they prevented models from properly converging. Then, we tested the correlation between female telomere change from emergence to parturition ($TL_{\text{birth}} - TL_{\text{emergence}}$) and female telomere change from mid lactation to weaning ($TL_{\text{weaning}} - TL_{\text{lactation}}$). This allowed us to determine if the magnitude of maternal telomere change over lactation was related to the magnitude of maternal telomere change at the start of the season, i.e. if females showing the greatest telomere loss during lactation were also those that showed the highest amount of telomere reconstruction prior to parturition.

Statistical analyses

All analyses were done in R v 4.0.2 (R Core Team 2021). We inspected model residuals for normality using density distribution, Q-Q plots, cumulative distribution functions and P-P plots obtained with the “fitdistrplus” package in R (Delignette-Muller & Dutang, 2015). Sample sizes are indicated as the number of observations (n) and number of individuals (N).
RESULTS

Telomere length repeatability and relationship with female age and body mass

Controlling for assay-related terms (plate and row within plate), the repeatability of female relative telomere length over the season was low, but significant ($r = 0.11$; CI$_{0.05} = [0.00, 0.26]$; $P = 0.044$; Fig 1). Relative telomere length was significantly and negatively associated with female age (LMM; $z$-age estimate $= -0.28$, CI$_{0.05} = [-0.51; -0.06]$, $t = -2.51$, $P = 0.013$), and positively associated with female mass ($z$-mass estimate $= 0.27$, CI$_{0.05} = [0.08; 0.46]$, $t = 2.79$, $P = 0.006$).

Seasonal changes in telomere length

Controlling for female ID, age, mass and assay-related terms in our model, female relative telomere length varied in a quadratic fashion with advancing breeding season, and differed between females that did or did not raise a litter (LMM; period x status interaction; $F_{3,107.6} = 3.16$, $p = 0.028$, $n = 154$ observations, $N = 43$ females; Fig 1, Table 1). For females that raised a litter, relative telomere length increased by 23%, though not quite significantly (Tukey HSD; $t = -2.34$, $p = 0.096$, Table 2), between emergence from hibernation to around the time of birth (i.e., during gestation). Relative telomere length then decreased significantly, by 28% between birth and weaning (Fig 1, Table 2). In females that did not raise a litter, relative telomere length also increased by 17% from emergence to what would correspond to around the time of birth for breeding females, and by another 10% to around the time of mid-lactation for breeding females (the increase from emergence to mid-lactation being significant; Fig 1, Table 2). In contrast to breeders, these females did not show a significant decrease in relative telomere length from the time of mid-lactation to the time of weaning of the offspring (Fig 1, Table 2). Interestingly, non-breeding females had significantly longer telomeres than breeding females.
from around the time of mid-lactation (+20%; Tukey HSD; t = -2.1, p = 0.04) to the time when breeding females weaned their offspring (+38%; Tukey HSD; t = -3.3, p = 0.001).

**Telomere length in relation to subsequent reproduction**

Controlling for maternal mass at emergence and assay-related terms, female telomere length at emergence from hibernation neither significantly affected the size (LMM; z-telomere length = 0.08; CI$_{95}$ = [-0.45; 0.60], t = 0.32, P = 0.753, N = 17 females) or mass (z-telomere length = 0.20; CI$_{95}$ = [-36.73; 31.13], t = 0.01, P = 0.991, N=17 females) of the litter at weaning.

Similarly, controlling for maternal mass, telomere change at the start of the active season (from emergence to birth; see Fig 1) did not significantly affect either litter size (z-telomere change = 0.11; CI$_{95}$ = [-0.65; 0.87], t = 0.32, P = 0.758, N = 16 females) or litter mass at weaning (z-telomere change = 30.28; CI$_{95}$ = [-17.47; 78.02], t = 1.41, P = 0.188, N = 16).

**Telomere loss following reproduction**

For females that raised a litter, telomere loss over lactation was significantly and negatively related to both litter size (LM; z-litter size = -0.28; CI$_{95}$ = [-0.53; -0.03], t = -2.38, P = 0.031; $R^2 = 0.23$; N = 17; Fig 2A) and litter mass at weaning (z-litter mass = -0.32; CI$_{95}$ = [-0.57; -0.08], t = -2.83, P = 0.013; $R^2 = 0.31$; N = 17; Fig 2B). Female telomere changes at the start and end of the breeding season were positively, but not significantly, correlated (Pearson’s $r = 0.39$; CI$_{95}$ = [-0.15; 0.75], t = 1.53, P = 0.149).

**DISCUSSION**

In Columbian ground squirrels, we found that telomere lengthening occurred between emergence of hibernation through to about the time of births, some 26 days later, both for breeding and non-breeding females (see Fig 1A). Afterwards, only breeding females
experienced significant shortening of their telomeres during the reproductive season, the amplitude of telomere erosion being negatively related to litter size and mass (Fig. 2).

It has been suggested that telomere lengthening at emergence from hibernation might occur to counteract the deleterious effects of hibernation (Hoelzl et al., 2016; Ruf & Bieber, 2020). The physiological costs of hibernation extend from impaired immune function (Cooper et al., 1992) to enhanced oxidative stress, the latter being particularly problematic during euther mic arousals associated with metabolic boosts and high production of reactive oxygen species (ROS) (Orr et al., 2009). Telomeres may be a target of ROS (von Zglinicki 2002, Reichert and Stier 2017; but see Boonekamp et al. 2017) and particularly vulnerable to rewarming processes during euther mic arousals, due to sudden increases in oxidative respiration (Murin et al., 2001). For instance, telomere shortening rates in hibernating edible or garden dormice were positively related to the time spent euther mic during the inactive period (Giroud et al., 2014), and to the frequency of arousals (Hoelzl et al., 2016). Reconstructing chromosome ends at the start of the active period would be of particular importance if long telomeres (or reduced annual shortening) are a seasonal prerequisite for individual survival (Bize et al., 2009; Wood & Young, 2019). Though this must still be determined in Columbian ground squirrels, associations between telomere length and survival have been documented across vertebrates (Haussmann et al., 2003; Tricola et al., 2018; Vera et al., 2012; Whittemore et al., 2019). Telomere maintenance (and rebuilding) may have coevolved with the relatively slow pace of life that is characteristic of some hibernating rodents (e.g., reduced mortality due to winter inactivity; Turbill et al. 2011, Constant et al. 2020). In our study, although most squirrels were captured on the day of emergence from hibernation (as known from the daily survey of the study site and by the condition of the animal upon capture), we cannot exclude the possibility that a few animals were caught slightly after the day of emergence. Thus, if anything, telomere reconstruction following hibernation may have been slightly
underestimated in the present study, and may be even more pronounced than our current data suggest.

In addition to counteracting the potentially deleterious effects of hibernation, findings of telomere lengthening at the end of hibernation and onset of the active season (Hoelzl et al., 2016; Ruf & Bieber, 2020; Turbill et al., 2013) question the importance of this process in determining reproductive success and individual fitness in the wild. In this regards, telomere elongation might be critical in at least two aspects: (i) by favoring reproduction, since greater telomere length (or reduced telomere erosion) has been associated with greater reproductive success (Angelier et al., 2019); or (ii) by serving as a pre-emptive change that mitigates the physiological costs of reproduction (i.e., shortened telomeres or higher telomere loss due to reproduction; Bauch et al. 2012, Sudyka et al. 2014, Bichet et al. 2020, but see Sudyka 2019).

In our study, we found no clear relationship between telomere lengthening at the start of the reproductive season (between emergence and birth) and female reproductive effort measured as the litter size or mass produced at weaning. These results suggest that telomere length at emergence of hibernation does not constitute a physiological constraint on reproduction for females in this species. This is perhaps not surprising, given that female Columbian ground squirrels are primarily income breeders: current reproductive effort depends more strongly on current environmental conditions (Dobson & Oli, 2001), than on carried over reserves and somatic condition from the previous year (Broussard et al., 2005; Rubach et al., 2016). In contrast, female telomere length of somatic cells decreased from lactation to weaning in breeding – but not in non-breeding (same relative chronological dates, see Methods) – individuals. In addition, in breeding females, telomere length decreased in relation to reproductive output: females producing larger and heavier litters experienced a higher telomere loss (see Figs 1 and 2).
Telomere loss may be the ultimate cellular consequence of increased oxidative metabolism and its inevitable production of reactive oxygen species ROS due to reproduction (Kirkwood and Kowald 2012; but see Speakman et al. 2015). Several studies have found faster telomere deterioration in breeding adults (Kotrschal et al. 2007, Reichert et al. 2014, Sudyka et al. 2014, 2019, Bichet et al. 2020), even if the link between oxidative stress and telomere loss remains unclear (Boonekamp et al., 2017). The present results seem to support the hypothesis that reproduction entails physiological costs such as increased telomere loss (but see Sudyka et al. 2019 for counter-examples). This is evidenced both by the difference between breeding and non-breeding females, and by the positive association between telomere loss and the increasing reproductive output by breeding females (i.e. litter size or mass).

Although ROS production during reproduction may contribute to shorter telomeres, other mechanisms, such as different changes in hormonal levels in breeding and non-breeding females across the reproductive cycle, might explain differential changes in telomere length. For instance, elevated testosterone has been found to increase telomere loss (Heidinger et al. 2021), whereas increased progesterone has been found to increase telomerase activation (Kyo et al., 1999). Both testosterone and oestrogen are likely to be higher in breeding females which are territorial during lactation (Murie and Harris 1988), and their effects on telomere dynamics (together with those of other hormones such as progesterone or prolactin) remain to be investigated.

Of interest is the result that telomere elongation prior to reproduction may perhaps play a role in mitigating the physiological costs of reproduction by pre-emptively reconstructing telomeres before the costly period of lactation, as we previously documented with regards to oxidative stress in this species (increased oxidative defences and reduced oxidative damage during lactation; Viblanc et al. 2018). We indeed found a positive correlation (r = 0.39), between pre-reproductive telomere elongation and post-reproductive telomere loss. However,
it important to note that the correlation was not significant, and additional data are therefore needed to conclude if the reproductive physiology of females is geared towards mitigating all of the physiological costs of lactation. One interesting question is how efficient this system actually is: previous studies have failed to evidence long-term fitness costs to reproduction in this species (Murie and Dobson 1987, Neuhaus 2000, Rubach et al. 2016; but see Neuhaus and Pelletier 2001). Variability in individual ability to compensate for somatic and telomere damage accumulated over hibernation also suggests that females of better individual quality may better buffer the negative effects of high reproductive investments (Angelier et al., 2019; Bauch et al., 2012; Sudyka, 2019). In line with this suggestion, we found a positive relationship between female emergence mass and telomere length. Such an observation is consistent with the idea that individual telomere length over the active period in female ground squirrels is partly explained by differences in individual condition or quality (Sudyka, 2019). More work is needed to clearly establish how far the consequences of reproduction on telomere length result from a trade-off between actual costs on one hand (telomere attrition due to reproduction) and variability in individual capacity to buffer these costs on the other.

Our findings add to the growing evidence that telomere length does not always reflect unidirectional cellular ageing processes in the organism, including in adult animals. We found that telomere dynamics in breeding females followed a seasonal pattern of reconstruction – and shortening as a cost of reproduction. Our results suggest (i) that telomere reconstruction occurs following hibernation in female Columbian ground squirrels, as in other hibernating mammals (ii) that telomere loss over during the breeding season at least partly reflects a physiological cost to reproduction, but (iii) we did not detect any effect of post-hibernating telomere elongation on future reproductive success. Disentangling the importance of the (likely co-occurring) processes of reproductive costs and variation in individual quality in buffering these
costs will require longitudinal surveys of life-long individual telomere dynamics and reproductive success in animals, indicating the necessity of long-term studies in the wild.

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**FIGURE CAPTIONS**

**Fig 1.** Relative telomere length dynamics in female Columbian ground squirrels over the course of the breeding season. Results are marginal means ± 95% confidence intervals. Differences between means were tested for relevant contrasts using Tukey HSD. Longitudinal differences are indicated Table 2. Cross-sectional differences between groups at specific time points are indicated by asterisks (**P < 0.01). Note that time intervals among sampling events are not identical (see Methods). Sample sizes are given below the means for breeding (black) and non-breeding (grey) categories. Relative telomere length was standardized (see Methods).

**Fig 2.** Relationship between relative telomere change over lactation and reproductive output in female Columbian ground squirrels, measured as (a) litter size and (b) total litter mass (g) at weaning (both variables standardized). The predictions and 95% confidence interval are given. Relative telomere length was standardized (see Methods).
Table 1. Mixed model estimates for the effects of breeding status (breeding [B] vs. non-breeding [NB]), period in the breeding season (emergence from hibernation, shortly before births, at mid-lactation and at weaning – see Methods) and their interaction on female relative telomere length in female Columbian ground squirrels. Individual ID, age, mass, and assay-related terms (plate, row within plate) were included as random factors in the models. Relative telomere length and independent variables are standardized (z-scores). N refers to individual and n to TL sample size (repeated measurements on individuals).

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Estimates</th>
<th>CI</th>
<th>t</th>
<th>p</th>
<th>n(N)</th>
<th>( R^2_{\text{max}}/R^2_{\text{cond}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>-0.50</td>
<td>-1.12 – 0.11</td>
<td>-1.62</td>
<td>0.108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period[Birth]</td>
<td>0.67</td>
<td>0.12 – 1.22</td>
<td>2.39</td>
<td>0.018*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period[Lactation]</td>
<td>0.33</td>
<td>-0.24 – 0.91</td>
<td>1.14</td>
<td>0.257</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period[Weaning]</td>
<td>-0.36</td>
<td>-0.91 – 0.19</td>
<td>-1.28</td>
<td>0.201</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Status[NB]</td>
<td>0.11</td>
<td>-0.48 – 0.69</td>
<td>0.36</td>
<td>0.721</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period[Birth] x Status[NB]</td>
<td>-0.15</td>
<td>-0.90 – 0.60</td>
<td>-0.40</td>
<td>0.693</td>
<td>154(43)</td>
<td>0.15 / 0.49</td>
</tr>
<tr>
<td>Period[Lactation] x Status[NB]</td>
<td>0.52</td>
<td>-0.25 – 1.30</td>
<td>1.34</td>
<td>0.183</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period[Weaning] x Status[NB]</td>
<td>0.87</td>
<td>0.11 – 1.63</td>
<td>2.27</td>
<td>0.025*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Tukey Honest Significant Differences for longitudinal mean comparisons in relative telomere length (standardized, z-scores) for female Columbian ground squirrels at different time points in the breeding season. Significant differences for $P < 0.05$ are indicated by asterisks.

<table>
<thead>
<tr>
<th>Status</th>
<th>Difference</th>
<th>Estimate ± SE</th>
<th>t ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeders</td>
<td>Emergence - Birth</td>
<td>-0.64 ± 0.27</td>
<td>-2.34</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>Emergence - Lactation</td>
<td>-0.31 ± 0.28</td>
<td>-1.10</td>
<td>0.689</td>
</tr>
<tr>
<td></td>
<td>Emergence - Weaning</td>
<td>0.34 ± 0.27</td>
<td>1.25</td>
<td>0.596</td>
</tr>
<tr>
<td></td>
<td>Birth - Lactation</td>
<td>0.32 ± 0.27</td>
<td>1.20</td>
<td>0.629</td>
</tr>
<tr>
<td></td>
<td>Birth - Weaning</td>
<td>0.98 ± 0.26</td>
<td>3.80</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>Lactation - Weaning</td>
<td>0.65 ± 0.27</td>
<td>2.46</td>
<td>0.073</td>
</tr>
<tr>
<td>Non-breeders</td>
<td>Emergence - Birth</td>
<td>-0.49 ± 0.25</td>
<td>-1.97</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>Emergence - Lactation</td>
<td>-0.81 ± 0.25</td>
<td>-3.17</td>
<td>0.011*</td>
</tr>
<tr>
<td></td>
<td>Emergence - Weaning</td>
<td>-0.48 ± 0.26</td>
<td>-1.87</td>
<td>0.246</td>
</tr>
<tr>
<td></td>
<td>Birth - Lactation</td>
<td>-0.32 ± 0.25</td>
<td>-1.27</td>
<td>0.585</td>
</tr>
<tr>
<td></td>
<td>Birth - Weaning</td>
<td>0.01 ± 0.25</td>
<td>0.05</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Lactation - Weaning</td>
<td>0.33 ± 0.26</td>
<td>1.29</td>
<td>0.571</td>
</tr>
</tbody>
</table>
**Fig 1.**

A graph showing the z-relative telomere length (T/S ratio) over different stages: Emergence, Birth, Lactation, and Weaning. The graph compares two statuses: B and NB.
Fig 2.
REFERENCES


Molbert, N., Angelier, F., Alliot, F., Ribout, C., & Goutte, A. (2021). Fish from urban rivers and with high pollutant levels have shorter telomeres. *Biology Letters*, 17(1), 20200819.


Murie, J. O., & Harris, M. A. (1988). Social interactions and dominance relationships


Viblanc, V. A., Schull, Q., Roth, J. D., Rabdeau, J., Saraux, C., Uhlrich, P., Criscuolo, F.,


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**DECLARATIONS**

The authors have no competing interests to declare that are relevant to the content of this article.