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RESEARCH ARTICLE

Tissue-specific telomere dynamics in hibernating arctic ground squirrels (*Urocitellus parryii*)

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ABSTRACT

Hibernation is used by a variety of mammals to survive seasonal periods of resource scarcity. Reactive oxygen species (ROS) released during periodic rewarming throughout hibernation, however, may induce oxidative damage in some tissues. Telomeres, which are the terminal sequences of linear chromosomes, may shorten in the presence of ROS, and thus the telomere length of an individual reflects the degree of accrued oxidative damage. This study quantified telomere length dynamics throughout hibernation in arctic ground squirrels (*Urocitellus parryii*). We hypothesized that telomere dynamics are tissue specific and predicted that telomere shortening would be most pronounced in brown adipose tissue (BAT), the organ that directly supports non-shivering thermogenesis during arousals. We used qPCR to determine relative telomere length (RTL) in DNA extracted from liver, heart, skeletal muscle (SM) and BAT of 45 juvenile and adult animals sampled either at mid- or late hibernation. Age did not have a significant effect on RTL in any tissue. At mid-hibernation, RTL of juvenile females was longer in BAT and SM than in liver and heart. In juvenile females, RTL in BAT and SM, but not in liver and heart, was shorter at late hibernation than at mid-hibernation. At late hibernation, juvenile males had longer RTL in BAT than did juvenile females, perhaps due to the naturally shorter hibernation duration of male arctic ground squirrels. Finally, BAT RTL at late hibernation negatively correlated with arousal frequency. Overall, our results suggest that, in a hibernating mammal, telomere shortening is tissue specific and that metabolically active tissues might incur higher levels of molecular damage.

KEY WORDS: Brown adipose tissue, Skeletal muscle, Oxidative damage, Biomarker, Reactive oxygen species, qPCR

INTRODUCTION

Hibernation is used by diverse species of mammals and one bird species to survive seasonal periods of food scarcity (Geiser, 1998; Staples, 2016). Hibernation is associated with longer maximum lifespans than predicted by body mass, a phenomenon that is supported by both predator avoidance and an overall slower pace of living (Turbill et al., 2011). If extrinsic mortality is diminished – as is the case when an animal is hibernating and protected from predation – selection should favor somatic maintenance (Kirkwood and Austad, 2000). One way to quantify the degree of incurred somatic damage and/or maintenance is via telomere length measurement (Monaghan and Haussmann, 2006; Monaghan, 2010). Telomeres are repetitive ($TTAGGG_n$ in vertebrates), nucleoprotein structures at the ends of linear chromosomes (Blackburn, 1991; Blackburn et al., 2015). These complex and highly regulated sequences protect genomic DNA by preventing chromosome end-to-end fusion and by buffering interstitial DNA from the 'end-replication problem', whereby the lagging strand loses terminal nucleotide bases with each cell division (Levy et al., 1992; de Lange, 2009; Greider, 2016). Telomeres can shorten as organisms age as a result of cell replication and in response to oxidative damage (von Zglinicki, 2002), and critically short telomeres can accelerate cellular aging by triggering cell senescence pathways (Collado et al., 2007). In terms of its ability to predict longevity or diagnose disease, telomere length is perhaps best viewed as an indicator, rather than a direct measure, of overall organismal health (Blackburn et al., 2015).

Hibernation in small mammals is characterized by two dramatically different physiological states: prolonged, multi-day torpor (metabolism depressed below basal rates and low body temperature) and brief (<1 day) intermittent arousals (rapid rises in metabolic activity and body temperature; Carey et al., 2003; Ruf and Geiser, 2015). During torpor, average heart rate across hibernators slows from 155 to 9 beats min^{-1} (Zatzman, 1984), core body temperature can drop as low as −2.9°C (Barnes, 1989) and cell division is arrested (Kruman et al., 1988; Wu and Storey, 2012). To fuel rewarming during periodic arousals from torpor, small mammalian hibernators increase their oxygen consumption by 300-fold over minimum hibernation levels (Karpovich et al., 2009). This dramatic surge in oxygen uptake, coupled with a pronounced increase in metabolic demand, can cause tissue-specific oxidative damage over an arousal episode (Carey et al., 2000; Orr et al., 2009). One organ that directly supports thermogenesis during arousal episodes is brown adipose tissue (BAT; Cannon and Nedergaard, 2004). In mice, heat production associated with uncoupling protein 1 (UCP1) activation in BAT mitochondria is accompanied by elevated levels of highly unstable reactive oxygen species (ROS; Chouchani et al., 2016), molecules that can interfere with and damage lipids, proteins and DNA. ROS produced in BAT mitochondria may damage telomeres by inducing DNA lesions, which can dramatically shorten telomeres over the subsequent cell cycle (von Zglinicki et al., 2000) and ultimately induce cell senescence (von Zglinicki et al., 2005). Although essentially all organs become more metabolically active over arousal episodes, BAT is the only organ to possess ROS-generating UCP1 (Rousset et al., 2004) and therefore has relatively higher potential to experience telomere shortening via oxidative damage. The other organ active in thermogenesis over periodic arousals is skeletal muscle (SM), which provides heat via shivering thermogenesis to fully raise core body temperature to euthermic levels (Allan and Storey, 2012; Staples, 2016).

The use of telomere length as a biological marker for cellular or organismal aging in hibernators has been investigated in three species: Djungarian hamsters (*Phodopus sungorus*; Turbill et al., 2012), garden dormice (*Eliomys quercinus*; Giroud et al., 2014) and

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List of abbreviations

BAT	brown adipose tissue
GSK3A	glycogen synthase kinase-3 alpha
LH	late hibernation
MH	mid-hibernation
qPCR	quantitative PCR
ROS	reactive oxygen species
RTL	relative telomere length
SM	skeletal muscle
UCP1	uncoupling protein 1

edible dormice (*Glis glis*; Turbill et al., 2013; Hoelzl et al., 2016a). In general, this work demonstrated that the use of torpor preserves telomere length, while arousal frequency and/or the number of arousals experienced throughout hibernation is negatively correlated with telomere length. In these studies, all measures of telomere length were from peripheral tissues (ear or buccal cells). While these tissues allow for minimally invasive and repeat sampling, ear tissue and cheek cells likely experience little cell turnover or ROS-mediated oxidative damage throughout hibernation and may not be reflective of telomere dynamics in other tissues. Therefore, it is worth investigating hibernator telomere dynamics in additional tissues – including those that experience a surge in ROS release over arousal episodes – to determine whether telomere length change is a local or systemic phenomenon.

In this study, we quantified relative telomere length (RTL) dynamics in four tissues (BAT, SM, liver and heart) in hibernating arctic ground squirrels (*Urocitellus parryii*). SM, liver and heart are appropriate comparison tissues to BAT in determining how ROS production might affect RTL. Unlike BAT, SM, liver and heart do not possess UCP1 in their mitochondria (Rousset et al., 2004). Although there is evidence that both BAT and SM release antioxidants during torpor in European ground squirrels (*Spermophilus citellus*; Vucetic et al., 2013), ROS production in BAT is higher than that in SM in mice (Mailloux et al., 2011) and rats (Schönfeld and Wojtczak, 2012); we presume a similar, tissue-specific ROS pattern is present in hibernating mammals. Additionally, the liver does not experience ROS-mediated oxidative damage during hibernation (Orr et al., 2009; Brown et al., 2012) and ROS production in the heart during late torpor is counteracted by antioxidants in the subsequent arousal episode (Wei et al., 2018). To test whether RTL shortened throughout hibernation, we sampled juvenile females at the middle (early January) and end (mid-March) of their hibernation season. As age and sex can affect telomere length dynamics (Monaghan, 2010), we also examined whether RTL differed among tissues across age and sex cohorts sampled late in hibernation. We hypothesized that hibernator RTL dynamics are tissue specific and predicted that RTL in BAT would exhibit the most pronounced shortening of the four tissues.

MATERIALS AND METHODS

Ethics statement

All animal trapping, housing, care and sampling was carried out in accordance with approved IACUC protocols (#1081763 and #340270) through the University of Alaska Fairbanks and with state (ADF&G #17-100) and federal (BLM #F-94817) permits.

Animals

Arctic ground squirrels, *Urocitellus parryii* Richardson 1825, are semi-fossorial rodents with a Holarctic distribution that includes Arctic Alaska (McLean, 2018). Maximum recorded age for arctic ground squirrels from our study region is 10 years for females and 6 years for males (S.M.W., C. E. Deane, B.M.B., G. A. Breed, C.L. Buck and C.T.W., unpublished data). All arctic ground squirrels used in this study were captured adjacent to the Dalton Highway near the Atigun River in northern Alaska (68°27′N, 149°21′W) in July 2017 and transported by truck to the University of Alaska Fairbanks. Prior to initiating hibernation, animals were individually housed in 48×32×32 cm hanging metal cages and provided with cotton bedding for nest construction. Initial ambient conditions were 20°C and 12 h:12 h light:dark photoperiod; 10 pellets per day of rodent chow (Mazuri, St Louis, MO, USA) and water *ad libitum* were provided throughout the active, pre-hibernation period.

Beginning 1 August 2017, animals were gradually transitioned (loss of 30 min of light per day) to a short photoperiod (4 h:20 h light:dark) to mimic arctic day lengths in autumn. Upon detection of hibernation readiness (e.g. not eating, quiet, curled up in nest) and before 1 December 2017, animals were moved to environmental chambers with an ambient temperature of 2°C and a 0 h:24 h light:dark photoperiod. Torpid animals were transferred into individual 43×27×19 cm plastic tubs (Nalgene, Rochester, NY, USA); food was withheld and gel packs (HydroGel, ClearH_2O, Portland, ME, USA) were provided for access to water. During hibernation, we monitored the animals by opening their cotton bedding and placing wood shavings on their exposed backs. We inspected daily to assess – by the presence or absence of shavings – the duration of torpor bouts and occurrence of arousal episodes (Pengelley and Fisher, 1961).

We used three groups of animals in this study: juvenile females (n=21), adult (>1 year) females (n=10) and juvenile males (n=14). The last two groups were from a concurrent experiment and were included opportunistically to augment late-hibernation samplings. To represent mid-hibernation (MH), 11 juvenile females were randomly selected and sampled in early January 2017. For late hibernation (LH), the remaining 10 juvenile females were sampled in mid-March 2017. Some adult females (n=7) and juvenile males (n=6) were sampled at LH, while the remainder were sampled 3 (n=2), 8 (n=3) or 15 days (n=6) after animals spontaneously ended hibernation (for sampling date details, see Table S1). For post-hibernation adult females and juvenile males, 10 pellets of rodent chow per day were provided from 3 days post-emergence to the day of sampling. Note: LH and post-hibernation adult females and juvenile males will hereafter be collectively designated as LH animals, as no significant differences in RTL were found between LH and post-hibernation in either age-sex group.

Tissue sampling

Approximately 18 h before sampling, we induced animals to begin arousing from torpor via 10–15 min of gentle hand manipulation before returning them to their nests. Immediately prior to sampling, aroused animals (core temperature >30°C) were anesthetized via exposure to isoflurane vapors (Isothesia, Henry Schein, Dublin, OH, USA). Animals were killed by decapitation before we excised approximately 1.5 g each of liver, whole heart, the quadriceps femoris muscle of the right hind leg and intrascapular BAT. The samples were then placed on RNase AWAY-treated foil (Thermo Fisher Scientific, Waltham, MA, USA), flash-frozen in liquid nitrogen, and stored at −80°C for later DNA extraction.

DNA extraction

We extracted DNA from liver, heart and BAT using a QIAamp Fast DNA Tissue Kit (Qiagen, Hilden, Germany). Following extraction, samples were stored in TAE buffer (Qiagen) at −80°C for later analysis. Prior to quantitative PCR (qPCR), all extracts were purified using standard ethanol precipitation procedures, as follows:

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50 µl of extracted DNA was combined with 150 µl of absolute ethanol, 5 µl of sodium acetate (3 mol l^{-1}, pH 5.2) and 1 µl of glycogen (all reagents from Thermo Fisher Scientific) and allowed to incubate overnight (at least 15 h) at −20°C. Samples were centrifuged for 30 min at 13,400 rpm. After this initial spin, the ethanol mixture was poured off, 500 µl of 75% ethanol was added, and the samples were spun at 13,400 rpm for 10 min; this wash step was repeated twice. After the final ethanol removal, samples were allowed to air dry for 10 min until the pellet was completely dry; 50 µl TAE buffer was then added to resuspend the DNA pellet.

Extract DNA concentration and purity were assessed using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific). We furthered verified DNA concentration with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). All samples used in this study contained at least 14.6 ng μl^{-1} DNA with quality ratios between 1.78 and 2.08 for A_{260}/A_{280} absorbance readings (a measure of protein contamination) and between 1.72 and 2.83 for A_{260}/A_{230} readings (a measure of phenol and/or salt contamination).

Telomere length assessment

qPCR was used to quantify tissue-specific telomere length in hibernating arctic ground squirrels. RTL was determined by measuring the factor by which an unknown sample differed from a standard sample in its ratio of telomere repeat copy number to non-variable gene copy number (non-VCN gene; Eqn 1). RTL is reflective of the average telomere length from the sampled tissue (Cawthon, 2002):

$$\mathrm{RTL} = \frac{E_{\mathrm{C}}^{C_{\mathrm{qC}}}/E_{\mathrm{T}}^{C_{\mathrm{qT}}}}{E_{\mathrm{SC}}^{C_{\mathrm{qSC}}}/E_{\mathrm{ST}}^{C_{\mathrm{qST}}}}, \quad (1)$$

where E is primer efficiency (expressed as 1+percentage efficiency; e.g. 98% efficiency is expressed as 1.98), C_q is quantification cycle, C is the non-VCN sample, T is the telomere sample, SC is the non-VCN standard and ST is the telomere standard.

All qPCR reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glycogen synthase kinase-3 alpha (GSK3A) was used as the reference non-VCN gene (tested for non-variability in copy number; after Smith et al., 2011). Primer sequences for the non-VCN gene were 5′-CTG ACA CTG CTG TCC TCA AG-3′ (GSK3A-F) and 5′-CGA TGG ACG AGG TAT AAT CA-3′ (GSK3A-R; Williams et al., 2011). Telomeric primer sequences were 5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′ (tel1b) and 5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3′ (tel2b; Epel et al., 2004). GSK3A and telomere qPCR assays were carried out in separate plates with 20 ng DNA, 400 nmol l^{-1} of each primer, 10 µl of Power SYBR Green Master Mix (Applied Biosystems) and 4.8 µl of molecular grade water (Thermo Fisher Scientific) per sample well. A standard curve with a five-step serial dilution starting at 20 ng μl^{-1} DNA was also run on each plate. Using the equation Efficiency=$-1+10^{-1/\mathrm{slope}}$, the slope of each run's standard curve was used to calculate primer efficiencies for each plate.

The thermal PCR profile for the GSK3A primers was 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 59°C and 20 s at 72°C. For the telomere primers, the thermal profile was 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 56°C and 20 s at 72°C. In each run, a final melting step was performed, ramping the temperature from 65 to 95°C in 1°C intervals. A no-template control (with molecular grade water) was included in each run. All samples and controls were run in triplicate. Hard-shell 384-well PCR plates (thin wall, skirted, clear; Bio-Rad, Hercules, CA, USA) and MicroAmp Optical Adhesive Film (Applied Biosystems) were used for all runs. See Table S2 for primer efficiencies and coefficients of variation per plate.

Statistical analyses

All analyses were performed in R (http://www.R-project.org/). Data are reported as means±s.e.m. We ran linear mixed models to test the following: (1) the effects of hibernation stage, tissue and the interaction between stage and tissue on RTL within juvenile females, (2) the effects of age, tissue and the interaction between age and tissue on RTL within LH females, and (3) the effects of sex, tissue and the interaction between sex and tissue on RTL within LH juveniles. In the case of a significant interaction, planned pairwise comparisons were run within each factor of the interaction. We used the Kenward–Rogers method to determine degrees of freedom, and the Tukey method for multiple comparisons to adjust P-values. Based on our finding that BAT and SM RTL differed between MH and LH squirrels, we subsequently tested the effect of arousal frequency on BAT and SM RTL in LH animals using a linear model; age and sex were included as additional factors. Finally, we used Pearson's correlation tests to determine whether RTL was correlated across tissues. We performed these correlation analyses via two approaches: (1) by including all animals and (2) within a group (e.g. MH juvenile females).

RESULTS

Hibernation in arctic ground squirrels

Hibernation duration ranged from 79 to 196 days. Juvenile females were sampled 90.5±1.6 days (MH group) and 166.5±1.7 days (LH group) after initiating hibernation. Adult females were sampled after 168.5±4.5 days of hibernation and juvenile males after 149.9±3.0 days. The average number of arousals throughout hibernation per group was as follows: 6.5±0.2 for MH juvenile females, 11.5±0.2 for LH juvenile females, 11.4±0.4 for adult females and 10.3±0.2 for juvenile males. Generally, animals aroused at similar frequencies (per month): 2.1±0.1 for MH juvenile females, 2.1±0.02 for LH juvenile females, 2.0±0.05 for adult females and 2.0±0.03 for juvenile males. Arctic ground squirrels spent 91.8±0.04% of the hibernation period in torpor.

Effects of stage, age and sex on RTL

We quantified RTL dynamics in four tissues from three groups of arctic ground squirrels: juvenile females at both MH and LH (stage effect), adult females at LH (age effect) and juvenile males at LH (sex effect). In juvenile females, RTL was shorter in BAT (P<0.001; Fig. 1) and in SM (P=0.01; Fig. 1) at LH compared with MH. Additionally, BAT RTL was longer than heart (P<0.001) and liver (P<0.001) RTL at MH. There was no difference in heart RTL (P=0.58) or liver RTL (P=0.19) between hibernation stages (Fig. 1). In LH females, there were no significant interactions between tissue type and age (P>0.05; Fig. 2). Juvenile males sampled at LH had longer RTL in BAT and SM than in heart (P<0.001 and P<0.01, respectively) and liver (P<0.001 and P<0.01, respectively; Fig. 3). Finally, at LH, BAT RTL in juvenile males was longer than BAT RTL in juvenile females (P=0.01); this difference between the sexes was not seen in SM (P=0.12; Fig. 3).

BAT RTL is shorter with higher arousal frequency

We found a significantly negative relationship between average monthly arousal frequency and BAT RTL (P=0.001 via linear model; n=34; Fig. 4) in LH animals. Additional factors age (P=0.45) and sex (P=0.08) did not impact BAT RTL. In SM, the relationship between arousal frequency and RTL was not significant

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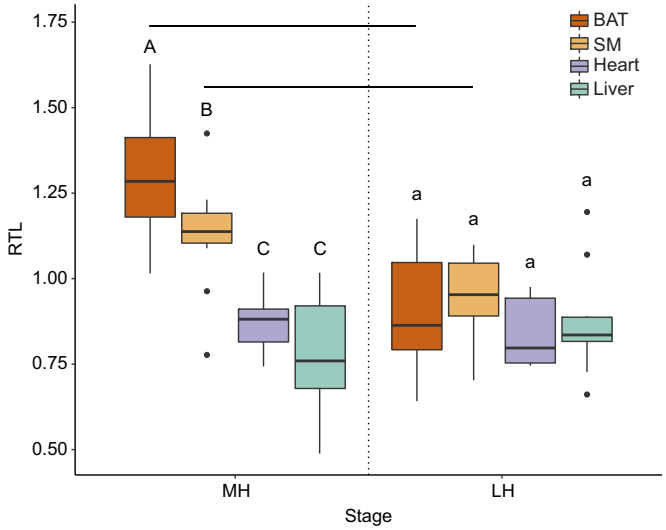

Fig. 1. Relative telomere length in juvenile females at mid- and late hibernation. At mid-hibernation (MH), relative telomere length (RTL) in brown adipose tissue (BAT) was greater than that in skeletal muscle (SM; P=0.02 via linear mixed model), heart (P<0.001) and liver (P<0.001). RTL in SM was also greater than that of heart (P<0.001) and liver (P<0.001). Late hibernation (LH) BAT and SM RTL was shorter than that at MH (P<0.001 and P<0.01, respectively). n=11 juvenile females were sampled at MH and n=10 at LH. Box plots show median (center line), upper and lower quartiles, and maximum and minimum values (whiskers); circles indicate outliers. Uppercase letters indicate significant differences between tissues at MH, and lowercase letters indicate significant differences between tissues at LH. Bars indicate significant differences between tissues across hibernation stage.

(P=0.78); age (P=0.78) and sex (P=0.08) also did not have significant effects on RTL in SM. See Table S3 for correlations between hibernation parameters.

No correlation in RTL among tissues

When including only LH animals (to account for the effect of stage), we found no significant correlations in RTL between tissues, save for the correlation between SM and BAT RTL, which was

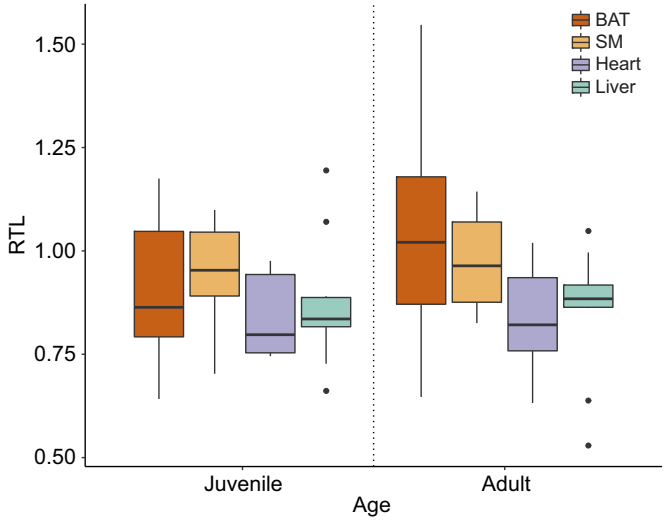

Fig. 2. RTL in juvenile and adult females at LH. A linear mixed model was used to test for differences in RTL between tissues within an age group and within a tissue across age groups. n=10 juvenile females and n=10 adult females were sampled at LH. There was no significant tissue×age interaction in LH females and no significant differences between planned comparisons.

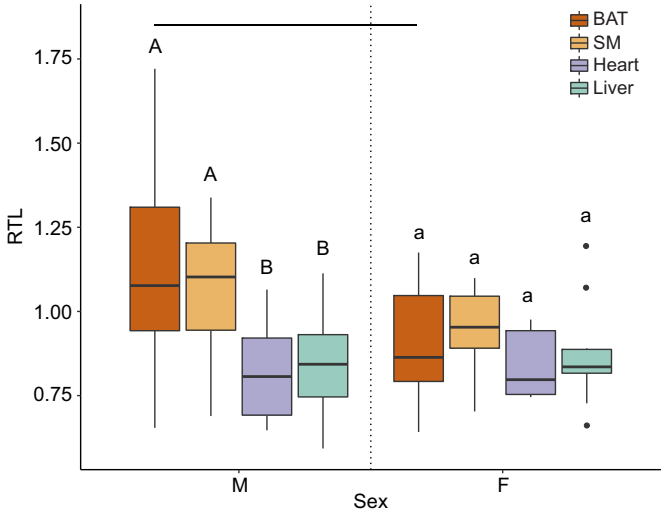

Fig. 3. RTL in male and female juveniles at LH. RTL of BAT and SM was greater than that of heart (P<0.001 and P<0.01, respectively, via linear mixed model) and liver (P<0.001 and P<0.01, respectively) in juvenile males. When comparing across sexes, BAT RTL was greater in males than in females (P=0.01). n=10 juvenile females (F) and n=14 juvenile males (M) were sampled at LH. Uppercase letters indicate significant differences between tissues in males, and lowercase letters indicate significant differences between tissues in females. Bar indicates a significant difference between BAT RTL across sexes.

positive. This pattern was maintained when including MH animals (Table 1).

DISCUSSION

Although hibernation is an effective and widely used survival strategy in mammals, it comes at a cost: hibernators experience

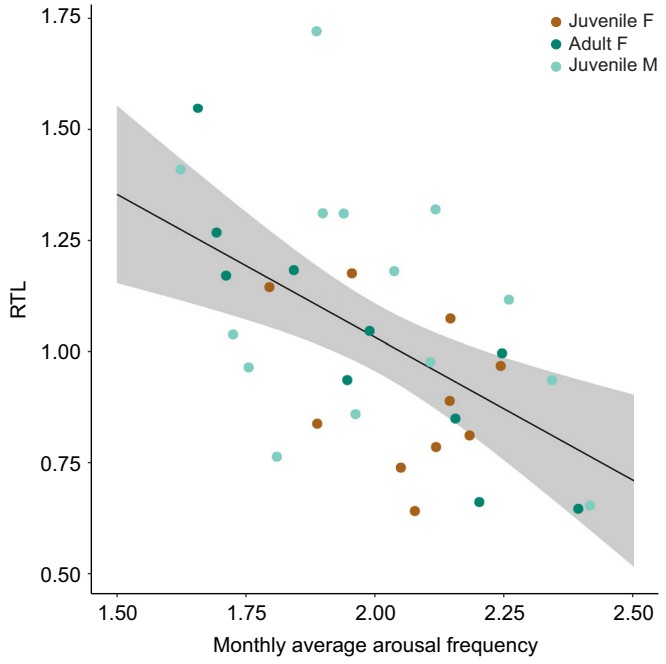

Fig. 4. Impact of arousal frequency on RTL in BAT at LH. Arousal frequency had a significantly negative effect on RTL in BAT (P=0.001 via linear model) in n=34 arctic ground squirrels at LH. Solid line represents the line of best fit for the linear model BAT RTL~Age+Sex+Arousal frequency. Shaded area represents the 95% confidence interval.

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Table 1. Pearson's correlations in relative telomere length (RTL) between tissues for all animals

	Liver RTL	BAT RTL	Heart RTL	SM RTL
Liver RTL	1	−0.18 (0.23)	−0.01 (0.95)	−0.07 (0.63)
BAT RTL	–	1	−0.01 (0.93)	**0.43 (>0.01)**
Heart RTL	–	–	1	0.19 (0.22)
SM RTL	–	–	–	1

Correlation values (*r*) are reported, followed by *P*-values in parentheses. All animals [mid-hibernation (MH) juvenile females and late hibernation (LH) juvenile females, adult females and juvenile males] are included. Significant correlations are in bold. Exclusion of MH juvenile females (to account for effect of stage) had no significant effect on the results, save for skeletal muscle: exclusion of MH juvenile females produced slightly less significant results (r=0.36, P=0.04). SM, skeletal muscle; BAT, brown adipose tissue.

ROS-mediated oxidative damage in some tissues over arousal episodes (Carey et al., 2000; Orr et al., 2009), which may be reflected in an individual's telomere length. To complement past studies that have explored the effects of hibernation on RTL in a single peripheral tissue, we thought it relevant to measure RTL in multiple internal tissues, and found compelling evidence for tissue-specific RTL shortening in hibernating arctic ground squirrels. In particular, BAT and SM RTL in juvenile females was dramatically shorter at LH than at MH. Additionally, RTL in BAT and SM was significantly longer than that in liver and heart at MH. In contrast, liver and heart RTL was very similar between MH and LH in juvenile females, further highlighting the tissue-specific nature of RTL shortening in arctic ground squirrels.

While this study focused on RTL shortening as a product of metabolic activity and ROS-mediated oxidative damage, initial telomere research was driven by the potential usefulness of telomeres as biomarkers for aging rates and longevity (e.g. Harley et al., 1990, 1992; Rudolph et al., 1999; López-Otín et al., 2013). More recently, there has been an interest in quantifying telomere dynamics in non-model organisms, including hibernating mammals (e.g. Hoelzl et al., 2016a). These efforts have revealed the great biodiversity of telomere dynamics across organisms and have expanded our understanding of telomere biology beyond its implications for human health. While it has been shown that telomere dynamics can be specific to age, sex and species, universal mechanisms have also emerged from this body of work, including the deleterious effect of oxidative damage and the tissue-specific nature of telomere length change.

All previous studies that have sought to find a relationship between telomere length and hibernation have connected telomere dynamics with torpor use or arousal frequency, as these two physiological states differentially influence ROS production (Orr et al., 2009). Initial work suggested that torpor use confers a protective effect on RTL, with demonstrations of RTL stasis or even lengthening across the hibernation season in Djungarian hamsters (*P. sungorus*; Turbill et al., 2012) and edible dormice (*G. glis*; Turbill et al., 2013). Later work found evidence for telomere shortening as a product of arousal frequency and time spent euthermic. In garden dormice (*E. quercinus*) that displayed short arousal episodes during the first month hibernating, buccal cell RTL did not significantly change over the hibernation season. However, in individuals with long arousal episodes in the first month of hibernation, overwinter changes in RTL were negatively associated with time spent euthermic (Giroud et al., 2014). Hoelzl et al. (2016a) found a similar relationship between euthermia and RTL shortening in *G. glis*: RTL buccal cells significantly shortened over the hibernation season and the best predictors of this effect were arousal number and arousal frequency.

The current study adds an important component to prior work on telomere dynamics in hibernating mammals and provides additional support for the relationship between arousal frequency and telomere shortening. In our study, juvenile females sampled at LH had significantly shorter RTL in BAT than at MH, likely due to the greater number of arousals experienced by the LH animals. BAT, the organ responsible for thermogenesis at the initiation of an arousal episode, is highly metabolically active during these periods and experiences arousal-induced oxidative damage (Orr et al., 2009). This damage is likely due to the large quantities of mitochondrial ROS released in BAT upon thermogenic activation; the level of ROS released is presumably above the amount found in other active tissues (Chouchani et al., 2016). (ROS appear to be essential signaling molecules in supporting thermogenesis, to the degree that pharmacological depletion of mitochondrial ROS in BAT results in hypothermia upon cold exposure; Chouchani et al., 2016). In our study, not only was BAT RTL shorter at LH than at MH but also RTL in this tissue was much greater than in liver or heart at MH. This finding is particularly intriguing in that it suggests that arctic ground squirrels 'prepare' for significant and predictable hibernation-induced telomere shortening in this tissue. Finally, although Vucetic et al. (2013) found that antioxidant levels increase during hibernation (and upon cold exposure) in BAT, perhaps the release of ROS in this tissue during arousal in arctic ground squirrels overwhelms antioxidant protection, ultimately shortening telomeres in this tissue.

Although the pattern was slightly less dramatic than that seen in BAT RTL between stages, we also found that RTL in SM was shorter at LH than at MH, presumably due to ROS production associated with shivering thermogenesis during periodic arousals. Non-shivering thermogenesis can also occur in SM via sarcolipin uncoupling of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA); however, sarcolipin is substantially down-regulated during hibernation (reviewed in Oliver et al., 2019), and therefore we expect that this process is less important. Two studies (James et al., 2013; Vucetic et al., 2013) addressed the question of antioxidant release in SM during hibernation, and found that antioxidant capacity generally increases in hibernating and cold-exposed animals. Brown et al. (2012) determined that decreasing ambient temperature increases maximal ROS production (but not basal ROS production) at complex III of the electron transport chain. As we suggest for BAT, shivering thermogenesis in arctic ground squirrels over arousals may produce enough ROS to overwhelm antioxidant protection and shorten telomeres.

The difference in BAT and SM RTL between stages is made more significant by the fact that RTL did not appreciably change in liver and heart tissue, neither of which is expected to experience UCP1-induced ROS release as these tissues lack this protein in their mitochondria. In addition, Orr et al. (2009) found that oxidative stress markers in liver did not differ between torpid and aroused arctic ground squirrels, and that oxidative stress was not associated with torpor in several tissues (including liver). In heart tissue, ROS that accumulate during torpor are counteracted by antioxidants released during the subsequent arousal (Wei et al., 2018). Thus, previous work suggests that neither the liver nor heart experiences a significant, enduring ROS load that might shorten telomeres. It is also possible, however, that less dramatic changes in RTL occurred in these tissues that we were unable to detect using qPCR, an assay that is not as sensitive as other methods, such as fluorescence *in situ* hybridization coupled with flow cytometry (flow-FISH; e.g. Wang et al., 2018). There is an additional possibility that telomerase – the enzyme that lengthens telomeres – could be active in liver and heart

throughout hibernation. While telomerase activity in most human somatic cells is low (Cong et al., 2002), in somatic cells of smaller mammals (particularly rodents) the enzyme is comparatively more active, and its activity varies in a tissue-specific manner (Prowse and Greider, 1995; Seluanov et al., 2007; Gorbunova and Seluanov, 2009). To date, there has been one published study that directly measured telomerase activity in a hibernator: Wang et al. (2011) detected telomerase activity in two bat species (*Hipposideros armiger* and *Rousettus leschenaultia*) that differed in their use of hibernation. In both species, telomerase activity was higher in metabolically active tissues (liver, spleen and kidney). In the heterothermic species (*H. armiger*), telomerase activity was higher than in *R. leschenaultia*, and this difference was even more pronounced when *H. armiger* was hibernating (Wang et al., 2011). Although further studies are needed, this previous work in bats supports the idea that telomerase could be impacting telomere length in other hibernating species.

DNA repair throughout hibernation should also be considered for its potential impact on telomere length. ROS interact with the guanine bases of telomeres to produce 8-oxo-7,8-dihydroguanine, a lesion that can be removed and repaired via base excision repair pathways (Rhee et al., 2011; Wang et al., 2010; Fouquerel et al., 2016). Although studies on DNA repair in hibernators are very limited, it appears that DNA repair mechanisms are shut down during torpor (or upon hypothermia induction; e.g. Baird et al., 2011) and resumed upon arousal (Yancey, 2018). This pattern of repair shutdown agrees with the slowing or cessation of many other physiological and molecular processes during torpor and their resumption upon the next arousal cycle (e.g. cell division, mitosis, transcription/translation; reviewed in Carey et al., 2003). One exception to this rule was noted in Schwartz et al. (2013): DNA repair genes such as *RAD50* are elevated during torpor in the hypothalamus of *Ictidomys tridecemlineatus*. However, repair dynamics in one tissue do not necessarily imply similar dynamics in any other, and it remains to be seen how DNA repair mechanisms operate in other tissues across hibernation. In our study, perhaps repair of telomere lesions is occurring in arctic ground squirrel liver and heart over arousals, which would support the lack of RTL change we saw in these tissues, but any potential repair activity in BAT may be overwhelmed by the presumed release of ROS in this tissue alone.

In addition to the effect of hibernation stage on arctic ground squirrel RTL, we also considered the effect of age. In adult and juvenile females sampled at LH, we found no differences in tissue-specific RTL. This was surprising, as we anticipated older females would have shorter RTL in BAT due to ROS exposure across multiple hibernation seasons. This apparent preservation of telomere length could be due to active season telomerase activity: perhaps telomeres shortened over hibernation are restored by telomerase during the subsequent summer (for discussions of telomere elongation in *G. glis*, see Turbill et al., 2013; Hoelzl et al., 2016a; Hoelzl et al., 2016b), thus preparing BAT telomeres for another round of hibernation-induced shortening; this may also be the case for SM. We also included juvenile males in our study to investigate a potential sex effect on RTL. Juvenile males had significantly longer RTL in BAT and SM than in liver and heart at LH and longer BAT RTL than seen in juvenile females. This pattern was likely due to differences in hibernation duration between these two groups: females hibernated longer than males, a pattern also seen in free-living populations (Sheriff et al., 2011). Presumably, if males and females had hibernated for equal duration, we would not see tissue-specific differences in RTL between the sexes; overall, we cannot say with certainty whether rates of RTL shortening differ between the sexes.

Our finding that arousal frequency negatively correlates with RTL in BAT – in other words, the more frequently an animal aroused the shorter were its BAT telomeres – is important in that it pinpoints the arousal episode as the driving force for telomere shortening in BAT. This effect agrees with past hibernator telomere investigations that also found a negative effect of arousal frequency on RTL in dormice buccal cells (Giroud et al., 2014; Hoelzl et al., 2016a). Surprisingly, however, we found no evidence of a correlation between arousal frequency and SM RTL, despite the fact that SM RTL appears to decrease across hibernation. Finally, we found no significant positive correlations between RTL across tissues, save for that between BAT and SM RTL. This is likely explained by tissue-specific differences in both the degree of accumulated oxidative damage at telomeres and potential telomerase activity. Considering how hibernator tissues differ in their metabolic contribution to periodic rewarming, future investigations should take care in selecting tissues with the understanding that telomere dynamics in one do not necessarily represent those in another.

This study is the first to quantify telomere dynamics from multiple, internal tissues in a hibernating animal – including BAT, the organ that fuels non-shivering thermogenesis at arousal initiation – and expands previous hibernator telomere work to include a ground squirrel species that is adapted to extreme conditions and seasonality. Future studies should include measures of telomerase activity (sampling during torpor, over arousal episodes and over active seasons); additionally, obtaining direct measures of ROS would provide a more holistic picture of what is driving telomere length dynamics throughout hibernation. Considering the possibility of tissue biopsies, a longitudinal study of telomere dynamics (starting with animals at pre-hibernation and continuing throughout the season) in BAT and in SM would be useful in understanding precisely how telomere length changes in these thermogenic tissues within an individual.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.M.W., B.M.B., C.T.W.; Methodology: S.M.W., B.M.B., A.S.K., C.T.W.; Validation: S.M.W., A.S.K.; Formal analysis: S.M.W., A.S.K.; Investigation: S.M.W.; Resources: B.M.B., C.T.W.; Writing - original draft: S.M.W.; Writing - review & editing: S.M.W., B.M.B., A.S.K., C.T.W.; Visualization: S.M.W.; Supervision: B.M.B., A.S.K., C.T.W.; Project administration: C.T.W.; Funding acquisition: S.M.W., B.M.B., C.T.W.

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Supplementary information

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