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Always a price to pay: Hibernation at low temperatures comes with a trade-off between energy savings and telomere damage

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Abstract

We experimentally tested the costs of deep torpor at low temperatures by comparing telomere dynamics in two species of rodents hibernating at either 3 °C or 14 °C. Our data show that hibernators kept at the warmer temperature had higher arousal frequencies, but maintained longer telomeres than individuals hibernating at the colder temperature. We suggest that the high-energy demand of frequent arousals is counteracted by a lower temperature differential between torpid and euthermic body temperature and that telomere length is restored during arousals, when the body temperature is returned to normothermic values. Taken together, our study shows that hibernation at low body temperatures comes with costs on a cellular level and that hibernators need to actively counterbalance the shortening of telomeres.

Introduction

Torpor and hibernation are states of prolonged inactivity associated with reduced metabolic rate (MR) and body temperature (T_b) and are regarded as the most efficient energy saving strategy employed by mammals and birds [1]. Despite its many benefits [2, 3], it is also increasingly recognised that torpor use also comes with costs, such as reduced immune function [4], slowed reactions [5] and increased oxidative stress [6] [for more see 7]. Hibernating edible dormice and woodchucks with large energy reserves show shallower torpor bouts, i.e. arousing more often from hibernation and maintaining a higher T_b during torpor than animals in poor condition [8, 9], which suggests that the costs of torpor could be temperature dependent. Energy saved through torpor is greatest at low T_b [10] and arousals from torpor represent the largest energy expenditure during hibernation [11].

Frequent arousals lead to rapid depletion of energy reserves and the upregulation of MR is associated with the production of reactive oxygen species (ROS) [6] that causes telomere shortening via DNA breaks [12-14]. Telomeres are noncoding, repetitive sequences of DNA at the end of chromosomes, which, together with telomere-associated proteins, prevent the degradation of the coding DNA during replication. Telomere length is often used as a marker of somatic maintenance and aging [15]. Telomeres shorten after each somatic cell division, i.e. mitosis, but telomere attrition can be accelerated by oxidative stress [14]. If telomere length is not restored, the cell eventually dies [16, 17]. During hibernation, mitosis is arrested at low temperatures and therefore telomere degradation is paused [18]. Hibernating at high T_b increases the frequency of arousal [19] and may increase the rate of telomere shortening [12-14]. However, if torpid T_b is near euthermic T_b then the associated increase in MR during frequent arousal may be less detrimental than fewer arousals from lower T_b

To test our prediction that individuals hibernating at warmer temperatures show less

RTL shortening over winter than animals hibernating at low T_bs, we performed a laboratory experiment investigating hibernation patterns (i.e. torpor bout length and arousal frequency) and relative telomere length (RTL) in edible dormice (*Glis glis*) and garden dormice (*Eliomys quercinus)*, hibernating at 3 °C or 14 °C.

Material & Methods

Experimental design

Experiments were carried out over 19 weeks (October 2016-March 2017) with 32 garden dormice and 15 edible dormice at the Research Institute of Wildlife Ecology, University of Veterinary Medicine, Vienna, Austria (48.22° N, 16.28° E). In total 16 garden dormice and 7 edible dormice were kept at 3 °C and 16 garden dormice and 8 edible dormice were kept at 14 °C. The experiment was split into three periods of 5-7 weeks (Table S1) to allow regular sampling points between periods. Individuals were weighed, and DNA samples were taken at the start and end of the experiment as well as in between periods. We estimated RTL by a quantitative PCR technique (see Supplementary Material) using DNA extracted from the inner cheeks by gently twisting a small brush for ca. 30 s inside each cheek. During the entire experiment, recording of nest temperature were used as a proxy for T_b to estimate torpor use, frequency of rewarming from torpor (arousal) and length of interbout euthermia (IBE), as described by Willis et al. (2005) (Supplementary Material, Fig. S1). Only torpor bouts >24 h were counted for calculation of mean torpor bout duration (TBD). We also measured MR in a subset (N=6 at each temperature) of garden dormice during periods 1 and 2 (see Supplementary Materials), but not in edible dormice.

Since hibernation at warmer temperatures is known to be associated with increased body mass loss [20], body mass loss was tightly monitored and body mass <70 g was used as the threshold to stop the warm temperature treatment. Nevertheless, one garden dormouse died unexpectedly at the end of period 2. We excluded seven further garden dormice of the 14 °C group, which had a low body mass, from the experiment after period 2 and allowed all remaining eight individuals of the former 14 °C-group and all 16 animals of the 3 °C-group to continue hibernation at 3 °C until the end of the experiment (Table S1). For the edible dormice, all 14 °C animals were excluded after period 1, but we continued the trials for the 3 °C animals, which were transferred from 3 °C to 22 °C (21.8 ± 0.1 °C (SE)) in period 3 (food and water provided *ad libitum*).

Statistical analyses were conducted using R (Version 3.3.1) [21]. Our sample size for garden dormice at 14 °C was reduced due to the death of one dormouse (excluded from all analysis, including MR), a data logger failure and thus no available torpor parameter for this individual and inefficient amounts of DNA for one individual (no RTL). Linear models were used to test for initial differences between the groups for RTL and body mass (all animals), and to test for differences in total MR (only garden dormice: $N_{3^{\circ}C}=6$, $N_{14^{\circ}C}=5$) caused by the temperature treatment and/or period. Linear mixed effects models were used to test time (time points 1,2,3, i.e. periods 1, 2) and temperature effects, and their interaction, on IBE duration, arousal frequency, TBD, body mass, MR and RTL, followed by ANOVA [22, 23]. To adjust for repeated measurements, we included individual as a random effect, but not state (torpid/euthermic), as this random factor increased the model Akaike's Information Criterion (AIC) [24] corrected for small sample sizes (AICc [25]) (see Supplementary Methods). The same approach was used to test the effect of arousal frequency on body mass (garden dormice: $N_{3^{\circ}C}=16$, $N_{14^{\circ}C}=14$; edible dormice: $N_{3^{\circ}C}=7$, $N_{14^{\circ}C}=8$). For statistical analyses of RTL (garden dormice: $N_{3^{\circ}C}=16$, $N_{14^{\circ}C}=13$; edible dormice: $N_{3^{\circ}C}=7$, $N_{14^{\circ}C}=8$), we [26] included initial RTL as a covariate to correct for the "regression to the mean" [12]. To evaluate whether RTL had increased or decreased following temperature treatment, we used paired t-tests. For change in RTL we selected best models using AICc. Variables tested were arousal frequency, TBD, body mass loss and IBE duration. Because of the limited sample size, we only used models with a maximum of three predictors. To analyse MRs we used total MR per animal as the response variable and included body mass as a covariate. Mass-specific MRs are given for descriptive purposes but were not used in statistical analyses.

Results

Temperature significantly affected RTL of garden dormice (Fig. 1a; temperature x sampling point, χ^2=5.16, df=1, p=0.023): Whereas RTL of the 3 °C-group significantly shortened over the first two periods (t=3.79, df=15, p<0.01; mean: -0.10 ± 0.03), RTL remained unchanged in the 14 °C-group (t=0.78, df=13, p=0.45 mean= -0.02 ± 0.03). Individuals at both temperatures showed an elongation of RTL in period 3 at 3 °C of 15 % (14 °C-group) and 7 % (3 °C-group), respectively (Fig. 1a). In edible dormice, RTL change was also significantly influenced by temperature (Fig. 1b; temperature x sampling time, χ^2=7.74, df=2, p=0.021; Fig. 1b). However, RTL had neither significantly shortened at 3 °C (t=1.31, df=6, p=0.237, mean= -0.32 ± 0.24), nor significantly increased at 14 °C (t=1.6, df=7, p=0.153, mean= 0.22 ± 0.14) after period 1. The individuals at 3 °C showed a rapid increase of RTL by 20 % in period 3 at 22 °C with food being available (Fig. 1b).

Nest temperature recordings showed a significant increase in arousal frequency and TBD at 14 °C for both species. While IBE was also significantly increased for garden dormice hibernating at 14 °C, IBE duration did not differ for edible dormice at both temperatures (Table 1). In both species, RTL change was best explained by arousal frequency (Table 2). Arousal frequency also significantly affected body mass in both species (edible dormice, 1 period: χ^2=20.79, df=1, p<0.001; garden dormice, 2 periods: χ^2=132.33, df=1, p<0.001).

Temperature treatments also influenced MR of garden dormice. During arousal, MR was higher in the 3 °C-group than in animals at 14° C (2.88 ± 0.19 $mlO_2g^{-1}h^{-1}$ vs. 2.22 ± 0.10 $mlO_2g^{-1}h^{-1}$). In contrast, MR during torpor was higher at 14 °C than at 3 °C (0.08 ± 0.01 $mlO_2g^{-1}h^{-1}$ vs. 0.05 ± 0.01 $mlO_2g^{-1}h^{-1}$). Because TBD was significantly shorter and arousal frequency and length of IBE were significantly higher in individuals at 14 °C (Table 1), total MR (individual mean calculated over the entire sampling period) was more than twice as high in the 14 °C-group than in the 3 °C-group (3 °C: 0.18 ± 0.04 $mlO_2g^{-1}h^{-1}$, 14 °C: 0.37 ± 0.01 $mlO_2g^{-1}h^{-1}$; χ^2=7.14, df=1, p=0.0075). Arousal frequency decreased again in 14 °C animals kept at 3 °C in the last period, while TBD lengthened and IBE duration consequently decreased (data not shown).

Discussion

Our study shows that individuals of both species hibernating at 14 °C spent more energy than their conspecifics hibernating at 3 °C but experienced less telomere attrition over the hibernation period. Our data do not only shed light on the observed trade-off between energy saving and preferred hibernation temperature in edible dormice [9] and woodchucks [8], but also support the idea that torpor is costly [7, 27].

Telomere shortening correlates with cellular oxidative damage [13] and thus can be seen as an integrative measure of oxidative stress, which is increased during rewarming [12]. While being torpid at 14 °C may be more energetically costly than at 3°C, rewarming from 14 °C requires a lower increase in MR, which is related to lower ROS production and therefore is likely to lead to less RTL shortening. Our data are consistent with the finding that telomere length is positively correlated with torpor frequency in Djungarian hamsters using daily torpor (T_b typically around 18 °C) [28]. Mitosis is arrested during torpor and the small increase of MR during arousals from high T_bs is unlikely to be associated with a pronounced production of

ROS, explaining why daily torpor is positively associated with RTL. Interestingly, this may provide an explanation for the abundance of daily heterotherms that do not reduce their T_b lower than 10°C, while hibernators, which allow their T_b to drop to near ambient temperature in deep torpor are less abundant in comparison [1].

Even without high intensity metabolic stress, RTL still decreases through high mitotic activity during arousals [18], suggesting the existence of a repair mechanism during arousal periods and/or also during torpor at warmer temperatures. This consideration is also in line with a study in which hibernation under fluctuating T_a (10-15 °C) in the laboratory did not lead to a decrease in RTL in garden dormice [29]. It has long been known that telomeres can be elongated mainly by the activity of the enzyme telomerase [30, 31] as well as by a DNA-recombination mechanism, i.e. alternative lengthening of telomeres [32]. Many small rodents express telomerase activity in cells of various tissues, including somatic cells [33]. A previous study found that telomerase activity in heart, spleen and kidneys was higher in hibernating than in active bats [34], although no information on T_b during hibernation was provided. Earlier work in ground squirrels has demonstrated that DNA [35], RNA [36], protein synthesis [37] and low levels of mitotic activity [38] can still take place at low temperatures, but will likely be drastically downregulated during torpor [39, 40] and only resumed during arousals.

Telomere elongation in edible dormice has so far been found in older individuals (≤ 41 %/year) [41], as well as in edible dormice that had a surplus of food (supplementary feeding of a free-ranging population/ food *ad libitum* in the laboratory) [12, 42]. In contrast, the observed increase of RTL in our study occurred in the 14 °C animals that had a higher energy demand than 3 °C animals during all periods and suggests a certain amount of plasticity in the maintenance of RTL throughout hibernation. Further, the maintenance of RTL through a lengthening mechanism also requires the mobilization of energy. In this study, energy originated exclusively from body energy reserves and our data indicate that RTL increase may be faster when food is provided, as seen by the rapid increase in RTL in edible dormice transferred from 3 °C to 22 °C. A similar strong increase in RTL has been found in food-supplemented edible dormice over 10 weeks [12]. These data support the hypothesis that telomere elongation is energetically costly [12]. The observed increase in RTL during the last period of hibernation in spring in both species suggests the existence of a predetermined seasonal, perhaps circannual program in hibernators. Dormice emerge from hibernation just before the start of the breeding season in mid to late spring (e.g. end of March in Northern Europe and in our colony). It has been shown that reproduction increases oxidative damage and/or telomere loss [43-45], suggesting that restoring RTL before the start of reproduction might be beneficial. The observed elongation just before the end of the hibernation season might also explain an earlier study that found that average telomere length did not shorten over the hibernation season in free-ranging edible dormice [42] (i.e. telomeres must have been either elongated at the end of the hibernation season - as found in this study - or at the beginning of the active season [41]).

In summary, our study suggests that deep hibernation comes with costs on a cellular level, i.e. increased telomere attrition, which has to be actively and energetically costly counterbalanced by the animals. Consequently, current estimates of the energetic savings during deep hibernation are likely overestimated.

Data accessibility

Data deposited in the Dryad repository: https://doi.org/10.5061/dryad.40br385 [46].

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Tables

Table 1: Comparison of torpor characteristics during the temperature treatment (Mean + SE). Mean torpor bout duration (TBD), mean interbout euthermia (IBE) and arousal frequency per week are shown over the total length of temperature treatments (T_a; 3 °C and 14 °C). Displayed values are calculated as average values of the individual means. Garden dormice were kept in two groups for 12 weeks (period 1+2, 3 °C: N=16, 14 °C: N=14), edible dormice only for 7 weeks (period 1, 3 °C: N=7, 14 °C: N=8).

			3 °C	**14 °C**	**Test results**
Garden dormice	Mean TBD (h)	P 1 + 2	239.4 ± 7.3	91.0 ± 4.2	T_a x period: χ^2=6.39, df=1, p=0.012
	Arousals/week	P 1 + 2	0.7 ± 0.1	1.7 ± 0.1	T_a: χ^2=144.6; df=1; p<0.001
	Mean IBE (h)	P 1 + 2	9.0 ± 0.3	11.1 ± 0.4	$T_{a:}$ χ^2=16.7; df=1; p<0.001
Edible dormice	Mean TBD (h)	P1	304.2 ± 17.5	181.5 ± 7.5	$T_{a:}$ χ^2=23.87, df=1, p<0.001
	Arousals/week	P1	0.4 ± 0.1	0.7 ± 0.1	$T_{a:}$ χ^2=13.13, df=1, p<0.001
	Mean IBE (h)	P1	12.7 ± 3.2	7.8 ± 1.4	$T_{a:}$ χ^2=2.29, df=1, p=0.130

Table 2: The three best candidate models explaining RTL after 12 and 7 weeks, respectively, in garden and edible dormice. All models were corrected for RTL1. Factors tested were arousal frequency, torpor bout duration (TBD), body mass loss (BMloss), interbout euthermia (IBE) and total metabolic rate (only for garden dormice).

	Model	**AICc**	**ΔAIC**
Garden dormice	**Arousal frequency + RTL1**	**-16.59**	**0**
	BMloss + RTL1	-16.24	0.35
	Metabolic rate + RTL1	**-15.93**	0.67
Edible dormice	**Arousal frequency + RTL1**	**78.99**	**0**
	BMloss + RTL1	84.23	5.24
	IBE + RTL1	85.27	6.28

Figure caption

Figure 1: Relative telomere length (RTL) over 4 sampling points (19 weeks) for (a) garden dormice and (b) edible dormice. Garden dormice were all kept at 3 °C during the last period; edible dormice at 22 °C. The 14 °C trial was ended after the first 7 weeks for edible dormice.