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

# Julia Nowack1,2\*, Iris Tarmann1\*, Franz Hoelzl3, Steve Smith3, Sylvain Giroud1, Thomas Ruf1

\* shared first authors

1 Department of Interdisciplinary Life Sciences, Research Institute of Wildlife Ecology, University of Veterinary Medicine, Vienna, Austria

2 School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK

3 Department of Interdisciplinary Life Sciences, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria

Corresponding author: JN; [j.nowack@ljmu.ac.uk](mailto:j.nowack@ljmu.ac.uk), +44(0)1512312415; <https://orcid.org/0000-0002-4512-5160>

**Supplementary Methods**

*Experimental design*

Experiments were carried out over 19 weeks between October 2016 and March 2017 with 32 sub-adult garden dormice (half of each sex) and 15 juvenile edible dormice (18 males, 4 females) and raised under natural climatic conditions in large outdoor enclosures at the Research Institute of Wildlife Ecology (FIWI, University of Veterinary Medicine, Vienna, Austria, 48.22° N, 16.28° E). These species are known to hibernate over a variety of temperatures [1-3]. Individuals of both species were not yet fully grown, i.e. garden dormice were born in 2016 and edible dormice, which read adult body size only at two years of age [4], were born in 2015. Individuals were relocated from their outside aviaries into the laboratory in autumn and placed into ventilated climate chambers (modified refrigerators, Liebherr GKv 5730). A total of eight animals were kept in each of the four climate chambers (separated by species), in standard holding cages (36Lx20Tx14H cm) that were connected to a nest box (plastic PVC tube; 110Dx17H cm). To mimic natural conditions during hibernation for the animals, the climate chambers were kept at constant dark and at a near constant temperature. Mean Ta of the climate chambers were measured via temperature loggers (resolution: 0.2°C, accuracy: ± 0.06°C; sampling interval: 60s), built at the Research Institute of Wildlife Ecology, and was 3.2 ± 0.1°C (SE) and 14.3 ± 0.1°C (SE). Edible and garden dormice gain large body fat stores during summer (~50% of their body weight) and usually rely on their fat reserves during the hibernation period [5-8]. Consequently, animals were not provided with food and water in the climate chambers.

In total 16 garden dormice and 7 edible dormice were kept at 3°C (approximately normal Austrian winter conditions) and 16 garden dormice and 8 edible dormice were kept at 14°C.

The experiment was split into three periods to allow regular sampling points between periods in which individuals were weighed and sampled for DNA (see Table S1). During the entire experiment, torpor patterns were assessed in all individuals (garden and edible dormice) via recording of nest temperature (see below), and metabolic rate was recorded in a subset (N=6 at 3°C, N=6 at 14°C) of garden dormice during periods 1 and 2 (see below for further details).

As hibernation at warmer temperatures is known to be associated with higher body mass loss [9], body mass loss was tightly monitored and body mass <70g was used as the threshold to stop the warm temperature treatment. Nevertheless, one garden dormouse died unexpectedly during period 2. We further excluded seven garden dormice of the 14°C group which had a low body mass from the experiment after period 2 and allowed all remaining garden dormice to hibernate at 3°C until the end of the experiment (N=16, N=8). 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); lower critical temperature of the thermoneutral zone of edible dormice [10]) in period 3. Animals kept at 22°C had access to food and water (apple and food pellets: ssniff®HA, ssniff GmbH, Soest, Germany). Animals were checked daily for activity and no individual entered hibernation at 22°C.

# **Table S1: Number of individuals per species and temperature treatment for the three periods (P1-3), including duration of each period.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Garden dormice** | | **Edible dormice** | |
| **P1** | 7 weeks | 3°C and 14°C (N=16, N=15) | 7 weeks | 3°C, 14°C (N=7, N=8) |
| **P2** | 5 weeks | 3°C and 14°C (N=16, N=15) | 7 weeks | 3°C (N=7; 14°C excluded) |
| **P3** | 7 weeks | All 3°C (N=16; N=8) | 5 weeks | 22°C (N=7) |
|  | **total 19 weeks** | | **total 19 weeks** | |

*Nest temperature*

Nest temperature was measured via customized temperature data loggers (resolution: 0.2°C, accuracy: ± 0.06°C; sampling interval: every minute), built at the Research Institute of Wildlife Ecology, and located at the bottom of each of the nest boxes, as previously described and used in garden dormice [11, 12]. Nests were large enough for a dormouse to fit completely inside but small enough to force it to sit directly on the data logger. The floors of the nests were covered with a thin layer of hay to provide familiar nesting conditions but to still ensure contact between the animal and data logger. We used nest temperature as a proxy for Tb to estimate torpor use of each dormouse, as described by Willis et al. (2005) (see Figure 1 in Supplementary Materials). In brief, nest temperature was used as an indicator of torpor patterns (torpor bout duration, arousal frequency and arousal length). Nest temperatures close to those of the cooling unit, i.e. below 5°C and 16°C, were used to identify torpor bouts. As we were not recording Tb, we could not differentiate between shallow and deep short torpor bouts and therefore only torpor bouts >24 h were counted for calculation of mean torpor bout duration (TBD). Short torpor bouts were only used by dormice at the settling in phase at the beginning of the experiment and not observed during the actual hibernation period. Temperatures clearly above these values indicated that the animal had aroused and was euthermic. Sudden temperature drops during arousals were ignored, as they did not indicate torpor use, rather they indicated that the animal had left the nest.

*Energy expenditure*

Energy expenditure was determined by measuring the rate of O2 consumption (VO2) using an O2 and CO2 analyzer (Servopro 4100, Servomex, Crowborough, UK) and mass flow meters (FMA 3100, Omega Engineering, Stamford, CT, USA) in a subset of 12 garden dormice (six from each temperature group; three animals of each group measured at the same time for one period). The metabolic chambers were connected to the analyzer (pull mode; order: metabolic chamber, pump, needle valve, flow meter, oxygen analyzer) with airtight tubes. Water vapor was removed from the air prior to analysis using a water trap. A gas switch allowed measurements of air from six metabolic cages and one reference air channel for one minute each, i.e., each individual was measured once every seven minutes. The oxygen analyzer was calibrated prior to the start of the measurement using a high precision gas mixing pump (H. Wösthoff, Bochum, Germany, type 55A27/7a). Air was continuously drawn through the cages with pumps at a flow rate of ~40 l h-1. VO2 was calculated by an R program using equation 10.6 by Lighton [13]. Mean metabolic rate during arousals was calculated as the average metabolic rate during arousals including rewarming from torpor, IBE and torpor re-entry; mean torpor metabolic rate was calculated from all consecutive values during deep torpor; total metabolic rate was calculated summing up all values measured for one individual, i.e. costs during torpor, rewarming, IBE and entry phase. For logistic reasons metabolic rate was measured only during period 1 and period 2.

*Body mass and DNA sampling for relative telomere length*

Individuals were weighed, and DNA samples were taken at the start and end of the experiment as well as in between periods. Initial body mass did not differ between individuals kept at 3°C and 14°C for either species, but was significantly lower for edible dormice kept at 22°C (garden dormice: F1,30=0.73, df=1, p=0.398; mean: 118.3 ± 2.3 g; edible dormice: F2,28=13.94, p<0.001; 3°C -14°C: post-hoc, t=1.21, p=0.458, mean: 101.9 ± 3.9 g; 22°C post-hoc, t≥3.59, p≤0.003, mean: 80.5 ± 1.3 g). DNA samples were collected with small brushes (gynobrush, Heinz Herenz Medizinalbedarf, Hamburg, Germany) from the inner cheeks by gently twisting the brushes for ca. 30s inside each cheek. The brushes were then separated from their stems, placed into Multi-SafeSeal Tubes (Carl Roth GmbH+ Co. KG, Karlsruhe, Germany) with 1ml BC buffer (EDTA, NaCl, Tris HCl; [14]), labeled, and stored at 3°C. DNA extraction was carried out the day after the cell collection with the DNeasy Blood & Tissue Kit (Qiagen, Germany). The manufacturer’s protocol was followed after pelleting the cells and removing the brushes. Relative telomere length (RTL) was measured with the quantitative polymerase chain reaction (qPCR) approach [15], adapted for garden dormice after [16]. In short, the PCRs were carried out in 20 µl reactions and all samples and controls were run in triplicate. Each reaction volume contained 400 nmol l−1 of each primer pair (Tel1b/Tel2b or c-MycF/c-MycR) and 10 µl of SensiFast SYBR no-ROX MasterMix (Bioline). PCR conditions on the Rotorgene Q quantitative thermocycler (Qiagen, Germany) for the telomere primers were 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 for edible dormice and garden dormice. For c-Myc, PCR conditions were 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 20 s at 61°C and 20 s at 72°C in edible dormice and 10 min at 95°C followed by 40 cycles of 10 s at 95°C, 20 s at 63°C and 20 s at 72°C in garden dormice. All telomere to non-variable copy number (non-VCN) gene ratios were normalized with a standard sample (RTL1) that was included in every run. Reactions were prepared in the Qiagility PCR robot (Qiagen, Germany) and an inter-assay calibrator and no-template controls were included in each run.

The assay used has been shown to be highly reproducible, see [17]. All samples from a single individual were included on the same PCR run so that we are sure that any detected difference is not due to run-to-run variation. We include a mixture of individuals from different treatments within the same run for the same reason. For the non-VCN gene and telomere reactions, mean qPCR efficiencies were 86.3% and 85.0% for garden dormice and 85.2% and 84.2% for edible dormice, respectively. The mean coefficient of variation among replicates (intra-assay variation) for cycle threshold (Ct) values of the non-VCN gene and telomere assay were 5.5% and 3.5% for garden dormice and 10.0% and 3.0% for edible dormice, respectively. The mean coefficient of variation for Ct values of the non-VCN gene among runs (inter-assay variation) was 3.1% and 4.7% (garden dormice) and 5.0% and 5.0% (edible dormice) for the telomere reaction.

*Data analyses*

Data are presented as mean ± standard error. N denotes the number of individuals. Statistical analyses for period 3 were conducted separately, as the sample size had been reduced and the Ta treatment modified so that all individuals of one species were kept at the same temperature.

Statistical analyses were conducted using R (Version 3.3.1). Normal distribution of residuals and homogeneity of variance were tested using Shapiro-Wilk tests and Levene’s tests (leveneTest in library ‘car’, [18]), respectively. We excluded the dead garden dormouse from all analyses. Furthermore, the amount of DNA for one garden dormouse at 14°C was not sufficient to analyze RTL, reducing the sample size at 14°C to N=14 (3°C: N=16); we had also measured metabolic rate in this individual reducing the sample size for MR at 14°C to N=5 (3°C: N=6). Due to a data logger failure we could not obtain the torpor pattern in one garden dormouse hibernating at 14°C, thus leaving us with an N=13 at 14°C for analyses of the relationship between RTL and torpor parameters (TBD, IBE, arousal frequency; 3°C: N=16). We were able to obtain data for torpor pattern and RTL for all edible dormice (3°C: N=7, 14°C: N=8).

Linear models were used to test for initial differences between the groups for RTL and body mass as well to test for differences in total metabolic rate caused by the temperature treatment and/or period (garden dormice).

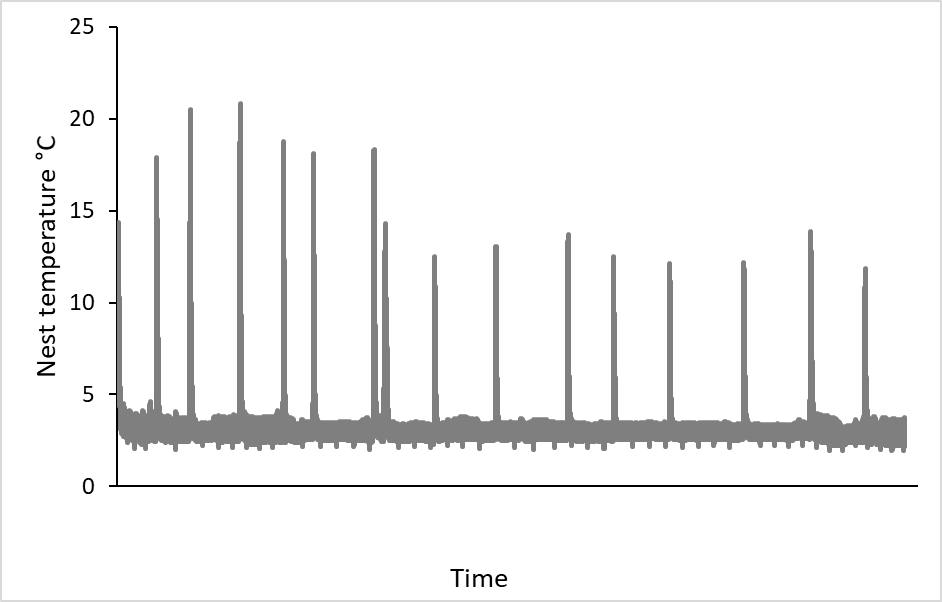
Linear mixed effects models were used to test time (sampling points 1,2,3) and temperature effects, as well as their interaction, on mean IBE duration, arousal frequency, mean torpor duration, body mass loss, MR and RTL, followed by ANOVA [18, 19]. The same was used to test the effect of arousal frequency on body mass. Individual ID was included as a random effect to adjust for repeated measurements. Mean IBE and mean torpor duration were calculated by excluding the data for the torpor bout before sampling/the IBE caused by sampling, respectively. However, these values were included in the total durations used for modelling.

For statistical analyses of RTL, we ran a second linear mixed-effects models with ‘ID’ and torpor phase [‘torpid’, ‘euthermic’ (n=5 for garden dormice, n=1 for edible dormice)] as nested random effects. We included initial RTL as a covariate to correct for the “regression to the mean” phenomenon in both models [16]. There was an unexpected significant group difference in initial RTL for garden dormice (t = 2.4515, df = 26.44, p = 0.021; 3°C: 1.07 ± 0.04 vs.14°C: 0.96 ± 0.03) that was also accounted for by including initial RTL length as a covariate into the models. When we compared the models with and without ‘torpor phase’ using Akaike’s Information Criterion (AIC) [20], corrected for small sample sizes (AICc in library ‘MuMIn’ [21]), we found that the models without this random effect had the lower AICc value (AICc ID as random effect vs. ID and torpor phase: garden dormice= -171.02 vs. -168.61; edible dormice= 54.85 vs. 58.62). Therefore, we used separate intercepts for each ID as the only random effect. Initial telomere length did not differ between groups for edible dormice (F2,28=0.9199, p=0.410). The statistical analyses of RTL were initially corrected for the qPCR runs; there was no significant difference between the qPCR runs, thus this variable was not included in further models. To evaluate whether RTL had increased or decreased at end of temperature treatment, we used paired t-tests.

Simple linear models were used to analyze metabolic rates because metabolic rate was measured in different individuals, not in repeated measurements. We used total metabolic rate per animal as the response variable and adjusted for body mass by including it as a covariate. Weight specific metabolic rates are given for descriptive purposes but were not used in statistical analyses.

For change in RTL we selected best models using AICc. Because of the limited sample size, we only used models with a maximum of three terms. All models were corrected for RTL1.Factors tested were arousal frequency, TBD, body mass loss and IBE and metabolic rate (only for garden dormice).

***Supplementary Figure***

******

Arousal

Torpor

October January March

**Figure 1: Nest temperature trace of one individual hibernating at 3°C over the entire experimental period.** Torpor bouts and arousal phases (including rewarming from torpor, interbout euthermia and re-entry into torpor) are indicated by the arrows.

**References**

1. Bertolino, S., C. Viano, and I. Currado, *Population dynamics, breeding patterns and spatial use of the garden dormouse (Eliomys quercinus) in an Alpine habitat.* Journal of Zoology, 2001. **253**(4): p. 513-521.

2. Moreno, S., *Reproduction of Garden Dormouse Eliomys quercinus lusitanicus, in southwest Spain.* Mammalia, 1988. **52**(3): p. 401-408.

3. Ruf, T., et al., *High survival in poor years: life history tactics adapted to mast seeding in the edible dormouse.* Ecology, 2006. **87**(2): p. 372-381.

4. Stumpfel, S., et al., *Differences in growth rates and pre-hibernation body mass gain between early and late-born juvenile garden dormice.* Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology, 2017. **187**(1): p. 253-263.

5. Bieber, C. and T. Ruf, *Summer dormancy in edible dormice (Glis glis) without energetic constraints.* Naturwissenschaften, 2009. **96**: p. 165-171.

6. Hoelzl, F., et al., *How to spend the summer? Free-living dormice (Glis glis) can hibernate for 11 months in non-reproductive years.* Journal of Comparative Physiology B, 2015. **185**(8): p. 931-939.

7. Schaub, M. and C. Vaterlaus-Schlegel, *Annual and seasonal variation of survival rates in the garden dormouse (Eliomys quercinus).* Journal of Zoology, 2001. **255**(1): p. 89-96.

8. Pajunen, I., *Body temperature, heart rate, breathing pattern, weight loss and periodicity of hibernation in the French garden dormouse, Eliomys quercinus L., at 4.2 ± 0.5 °C.* Annales Zoologici Fennici, 1974. **11**(2): p. 107-119.

9. Geiser, F. and L.S. Broome, *The effect of temperature on the pattern of torpor in a marsupial hibernator.* Journal of Comparative Physiology B, 1993. **163**(2): p. 133-137.

10. Heldmaier, G. and R. Elvert, *How to enter torpor: thermodynamics and physiological mechanisms of metabolic depression*, in *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium. Biological Papers of the University of Alaska*, B.M. Barnes and C. Carey, Editors. 2004, Institute of Arctic Biology, University of Alaska: Fairbanks, Alaska. p. 185-198.

11. Giroud, S., et al., *Late-born intermittently fasted juvenile garden dormice use torpor to grow and fatten prior to hibernation: consequences for ageing processes.* Proceedings of the Royal Society of London B: Biological Sciences, 2014. **281**(1797).

12. Mahlert, B., et al., *Implications of being born late in the active season for growth, fattening, torpor use, winter survival and fecundity.* eLife, 2018. **7**: p. e31225.

13. Lighton, J.R.B., *Measuring metabolic rates. A manual for scientists*. 2008, New York: Oxford University Press.

14. Thomas, P. and M. Fenech, *Chromosome 17 and 21 aneuploidy in buccal cells is increased with ageing and in Alzheimer's disease.* Mutagenesis, 2008. **23**(1): p. 57-65.

15. Thomas, P., N.J. O’ Callaghan, and M. Fenech, *Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer's disease.* Mechanisms of Ageing and Development, 2008. **129**(4): p. 183-190.

16. Hoelzl, F., et al., *Telomere dynamics in free-living edible dormice (Glis glis): the impact of hibernation and food supply.* The Journal of Experimental Biology, 2016. **219**(16): p. 2469-2474.

17. Hoelzl, F., et al., *Telomeres are elongated in older individuals in a hibernating rodent, the edible dormouse (Glis glis).* Scientific Reports, 2016. **6**: p. 36856.

18. Fox, J. and S. Weisberg, *An companion to applied regression*. 2 ed. 2011, Thousand Oaks, CA: Sage.

19. Pinheiro, J., et al., *Linear and nonlinear mixed effects models*. 2014.

20. Akaike, H., *A new look at the statistical model identification.* Automatic Control, IEEE Transactions on, 1974. **19**(6): p. 716-723.

21. Barton, K. *MuMIn: Multi-Model Inference. R package version 1.15.6*. 2016; Available from: <https://CRAN.R-project.org/package=MuMIn>.