Aus dem Department für Integrative Biologie und Evolution
der Veterinärmedizinischen Universität Wien

Konrad-Lorenz-Institut für Vergleichende Verhaltensforschung
(Leiter: Univ.-Prof. Leonida Fusani, PhD.)

„Variation in telomere length across tissue types in edible dormice *(Glis glis)*“

Diplomarbeit

zur Erlangung der Würde einer

MAGISTRA MEDICINAE VETERINARIAE

der Veterinärmedizinischen Universität Wien

vorgelegt von

Clara Noé-Nordberg

Wien, im April 2020

Betreuer:

Steven Smith, PhD.
Konrad-Lorenz-Institut für Vergleichende Verhaltensforschung
Department für Integrative Biologie und Evolution
Veterinärmedizinische Universität Wien

Begutachter:

Priv.Doz. Dr.phil. Claus Vogl
Institut für Tierzucht und Genetik
Department für Biomedizinische Wissenschaften
Veterinärmedizinische Universität Wien

Acknowledgement

I am deeply grateful to my supervisor Steven Smith, PhD for supporting the idea and implementation of my thesis. His inputs and suggestions for improvement were an enormous help and have enabled this project to lead to good results.

I want to offer my special thanks to Franz Hölzl, PhD. He answered every question concerning lab work as well as writing and was a great and very patient help over the last few months. Steve and Franz definitely aroused my enthusiasm for genetics and research.

I also want to thank the whole team of the Genetics Lab of the Konrad-Lorenz-Institute for answering every question and helping me to find my way in the lab, but also for accepting me as a part of the team over the last months.

Last, but not least, I want to thank my family for supporting me over the last years. I am very grateful to have such generous, devoting and loving parents who are always backing me.

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1 INTRODUCTION

1.1 Telomeres and Telomerase

1.1.1 Structure and Function

Since 1930, it is known that chromosomes have special structures on their ends that ensure stability and prevent breaking or fusing of the chromosome (McClintock 1939, McClintock 1941). Each chromosome of humans and other animals has a highly repetitive non-coding sequence of DNA at its end that forms, together with associated proteins, the telomeric structure and prevents loss of genetic information. Due to the end replication problem, telomeres shorten with every cell division until a critical short telomere length is reached and the cell stops dividing and enters a senescence phase (Blackburn 1991, Hoelzl, Smith et al. 2016).

The DNA polymerase, which is responsible for building a complementary strand of the DNA molecule in the course of mitosis, adds deoxynucleotide phosphates (dNTPs) from the RNA primer marked 5' end to the 3'end of the chain. While replicating the 3'-5' strand of the DNA, which is called the "leading strand", the polymerase can proceed continuously to the very end of the chromosome, whereas the 5'-3' "lagging strand" gets replicated discontinuously in small fragments (Okazaki-fragments). Due to the lack of sufficient template, the last piece of the lagging strand remains unreplicated and a shortening of chromosomes and a loss of genes is the consequence. (Petkova et al. 2011)

To protect the coding genes, short sequences of DNA, in mammals 5'-TTAGGG-3', provide a cap on each end of the chromosomes and are called telomeres (Meyne et al. 1989). With every cell division, instead of the coding genes, the telomeres get shorter which ultimately results in cell senescence. The enzyme telomerase or the ALT (alternative lengthening of telomeres) mechanism however, are known to restore telomere length in certain cell types (Arsenis et al. 2017, Hoelzl, Smith et al. 2016). Apart from maintaining chromosome length, telomeres also prevent end – to – end fusion or modifications of the chromosomes (Martínez and Blasco 2017).

The telomere sequence is bound by the telosome or shelterin complex, a protein complex consisting of six proteins with different functions. They are, among other things, responsible for monitoring and regulating telomere length through negatively regulating telomerase and protecting the end of telomeres by suppressing DNA damage responses at chromosome ends. (Armanios and Blackburn 2012, Xin et al. 2008)
Apart from the end replication problem, telomere shortening also occurs as a result of oxidative stress, a surplus of free oxygen radicals caused by inflammation, infections or environmental exposures. When not counteracted by antioxidant defences, the associated telomere shortening can lead to many degenerative diseases and cancers (Barnes et al. 2019).

Telomerase, a ribonucleoprotein consisting of a protein that carries the reverse transcriptase of the telomere, an RNA component, and some other proteins (for example dyskerin), is able to attach to the single stranded end of the molecule and effect an elongation of the 3' end of the chromosome (Armanios and Blackburn 2012, Janning and Knust 2008). In humans, active telomerase is only found in gametes and stem cells as well as in tumour cells (Zvereva et al. 2010). In contrast, studies on mice and other rodents showed, that there is active telomerase in somatic tissue cells. This difference between humans and rodents could be explained by the differences in body mass. Higher body mass implies more cells and a higher mutation risk, so it is hypothesized that the suppression of telomerase in somatic cells works as tumour suppressor mechanism (Seluanov et al. 2006).

1.1.2 Measurement Methods

There are different methods to measure the length of telomeres. The analysis of terminal restriction fragmentation (TRF) consists of the digestion of DNA by restriction enzymes, that cannot recognize subtelomeric and telomeric regions and therefore do not cut them, and afterwards an agarose gel electrophoresis which presents telomeres as a smear (Kimura et al. 2010). A source of error for this method are the included subtelomeric regions, furthermore, a high amount of DNA needed and it cannot detect very short telomeres or the length of single telomeres. Among the alternatives for measuring telomere length are polymerase chain reaction-based techniques (PCR) such as quantitative PCR (qPCR) or monochrome multiplex

quantitative PCR (MMqPCR). The telomeric DNA, targeted by a specific oligonucleotide primer, is amplified by PCR and quantified by measuring the cycle number at which the fluorescent signal of an intercalating dye (SYBR green) begins to increase above a baseline threshold. The comparison of this telomere PCR cycle threshold to that of a single-copy (or non-variable in copy number (non-VCN)) gene provides information about the relative telomere length (RTL) (Cawthon 2002). To avoid errors due to uneven pipetting, both, non-VCN gene and telomere, are amplified in the same tube when one applies MMqPCR (Cawthon 2009). For all PCR techniques, only a small amount of DNA is needed. On top of the methods described, there are some more, very specific techniques, like single telomere length analysis (STELA) for critically short telomeres of a subset of chromosomes or quantitative fluorescence in situ hybridisation (Q-FISH) for chromosome imaging following hybridization. (Montpetit et al. 2014)

There are a variety of methodologies available to measure telomerase activity in cell lines (summarised in (Criscuolo et al. 2018). The most commonly used is the telomere repeat amplification protocol (TRAP). A synthetic telomerase substrate (primer) is added to a cell lysate where telomerase can extend this sequence. After a specific incubation time, the enzyme is heat inactivated and a PCR of the newly synthesised telomeric DNA is carried out. The result can then be visualized and quantified on a polyacrylamide gel. This gel-based method is not feasible for cell material that shows very low telomerase activity and was therefore adapted by (Ludlow et al. 2014), using droplet digital PCR (ddPCR). In ddPCR, the PCR mix is divided into thousands of droplets, emulsified in an oil matrix, followed by end-point PCR. The number of positive droplets, containing newly synthesised telomeric DNA, are counted via flow cytometry as a final step. Therefore, this method can be used to detect telomerase activity at levels as low as a single cell. (Ludlow et al. 2014)

1.1.3 Pathologies

In the 1980s, scientists recognized that telomere length is much shorter in somatic cells of adults than in sperm cells and identified telomeres as a marker for aging and replicative potential (Cooke and Smith 1986).

Critically short telomeres lead to replicative senescence and a lower capability of tissue renewal in stem cells, potentially leading to dysfunction. In humans, mutations of telomere maintenance genes, especially for shelterin complex proteins and telomerase, can lead to premature shortening and cause diseases including dyskeratosis congenita, bone marrow failure, aplastic anaemia, idiopathic pulmonary fibrosis and cancer. (Armanios and Blackburn 2012)
In mice, the effects of short telomeres and low telomerase activity are less distinctive, leading to infertility and seldom to cancer (Calado and Young 2012).
Disabled tumour suppressor genes (p53) can cause further expansion, continuous shortening, chromosomal instability up to uncapping of the chromosomes and end-to-end fusion. Cell death (mitotic catastrophe) could be a consequence but alternatively, under the presence of active telomerase or ALT, the cells can divide and transform to a cancerous state (Martínez and Blasco 2017).

Due to the frequency of occurrence of age-related diseases and cancer, and newly discovered effects across a broad range of species, research in telomeres and telomerase activity has a growing and important role for understanding developing processes and uncovering novel treatment methods.

1.2 The Edible Dormouse

The edible dormouse *(Glis glis),* a species of the family *Gliridae,* is a small nocturnal hibernator living in the deciduous woods of Middle and West Europe. In September, the edible dormice entrench themselves into soil to hibernate until May, subsisting only on their fat reserves. Reproduction normally takes place in summer but depends on food availability. They can even skip reproduction in years of low seed production due to the low survival expectation for offspring in the absence of beech or oak mast (Lebl et al. 2010). In summer, they build their nests in tree holes where they also raise their offspring.

For most species, telomeres shorten with increasing age. Interestingly, it was found that edible dormice, which are very long-lived proportional to their body size, are able to elongate their telomeres with increasing age after an initial decrease in telomere length in the first five years

of their life (Hoelzl, Smith et al. 2016). It has also been shown that telomeres in edible dormice get shorter over the hibernation season in winter - due to periodic re-warmings - but can be elongated again in summer if food availability is sufficient (Hoelzl, Cornils et al. 2016, Turbill et al. 2013).

Long telomeres, as measured in somatic cells such as buccal cells, could explain why this species is able to reach an age of 13 years, but the reason and mechanism for elongating their telomeres is not fully clear. As telomerase can be activated in somatic cells of small rodents, this enzyme could be responsible for the elongation. In other species, an elongation of telomeres is only detectable in cancer cells but it is likely that similar results would be detected in other species with no reproductive senescence. (Hoelzl, Smith et al. 2016)

2 AIM OF WORK

In this study, we want to test if there is variation in relative telomere length across different tissue types in edible dormice, and if this variation is related to specific mechanisms like cell turnover rate and exposure to oxidative stress. As cell types have specific functions and different turnover rates, as well as varying levels of exposure to oxidative stress, the effects of telomere shortening and degeneration are expected to be highly different across tissue types. These variations in tissue telomere length are expected, however, to be correlated among individuals. Knowledge about telomere length and shortening in different tissues could be beneficial for developing screening methods as well as specific medical treatment of degenerative diseases or cancer. It may also have an impact on sample selection and design of further studies. We concentrate on liver, muscle, brain, blood, skin and mucosa cells as test tissues.

It is known that telomerase is active in stem and germ cells, but also in tissue specific somatic cells in small rodents. As we assume that telomerase is responsible for telomere elongation we expect that this enzyme is active in the tested tissues and that levels vary among individuals and tissue types. Furthermore, we expect that there is a correlation between telomerase activity and telomere length.

3 MATERIAL AND METHODS

3.1 Material

The tissue samples were obtained from 20 yearlings of the edible dormice population bred at the Forschungsinstitut für Wildtierkunde in Vienna (FIWI), that were removed and euthanised as part of routine population management in autumn 2018. To assess differences in the relative telomere length (RTL) in various tissue types, samples of 6 tissues per individual were used – liver, muscle, blood, mucosa, brain and skin. For extraction of mucosa cells, buccal swabs (Gynobrush®) were applied and ear biopsies were used as skin samples. The organs were removed immediately and flash frozen on dry ice before storing them in the -80 degrees centigrade freezer. The sacrificing of the yearlings was approved by the ethics committee (permit number ETK-14/06/2018).

All qPCR reactions were prepared using Qiagility PCR robot (Qiagen, Germany) and the amplification was conducted by Rotorgene Q quantitative thermocycler (Qiagen, Germany).
For analysing the data, the software LinRegPCR (2012.0) was applied and the statistics were calculated with RStudio (RStudio, Inc.). For ddPCR, droplets were generated using QX200 droplet generator (Bio-Rad, California, USA) and read with QX200 droplet reader (Bio-Rad, California, USA). For all centrifugation steps an Eppendorf centrifuge 5415R (Eppendorf, Hamburg, Deutschland) was used.

3.2 Methods

3.2.1 DNA Extraction

First, DNA was extracted from the tissues. Small pieces (approx. 50 mg) of the samples were digested in 500 µl TNES buffer and 15µl proteinase K while shaking (600rpm) on the heat block (55°C). The TNES buffer consisted of 5M NaCl, 3M TrisHCl (pH 7.5), 20% SDS and 0.5M EDTA. For the mucosa samples, the brushes were removed after this step. Once

dissolved, 170 µl 5M NaCl were added and the samples were centrifuged for 20 minutes at 12.000 rpm. The supernatant was recovered into a new tube before adding 500 µl isopropanol and 1 µl Glycogen and incubating 30 min at -80°C. Subsequently, the samples were spun again for 20 minutes at 4°C. In the next step, the supernatant was discarded and the DNA pellet was resuspended with 750 µl 70% Ethanol before a further centrifugation for 20 minutes at 4°C. After discarding the supernatant for a last time, the DNA pellet was dried on the heat block at 37°C before resuspension with 50 µl TE buffer.
After extracting, the DNA concentration of the samples was measured via NanoDrop 2000c (Thermo Fisher Scientific, USA). All extracted DNA samples were diluted to 10 ng/µl using ddH_2O.

3.2.2 Quantitative PCR

The real-time quantitative PCR (qPCR) is an extension of the polymerase chain reaction (PCR), that enables quantification of the starting amount of DNA by measuring the cycle number at which the fluorescence increases above the baseline. As described in 1.1, it is necessary to apply a qPCR for telomeres and one for a single copy gene to measure the relative quantity (length) of telomeres per genomic equivalents. These values for each individual are then compared to a standard sample common to all runs and presented as a ratio of individual telomere length versus the standard telomere length.

Before running qPCR with all samples, multiple test runs on each tissue of sample number one were done in order to find out, which conditions and compositions work best for obtaining a single peak in the qPCR melt curve and for achieving an adequate efficiency of the amplification *(Tab.1 and Tab.2)*. The reproducibility was also tested by repeating the assay on the same extracts. The slope *y* was close to the value 1 and the reproducibility resulted in R^2=0.9505 *(Fig.1)*.

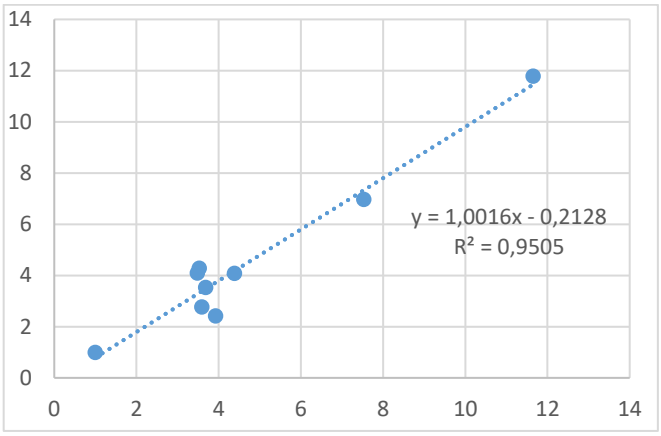

Fig. 1: Reproducibility

For marking telomeres, the primers Tel_1b (5'- CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3') and Tel_2b (5'- GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT -3') were used. The qPCR Master Mix consisted of 10 µl GoTaq® qPCR Master Mix (Promega), each primer in a final concentration of 0.6 µM, 200 µM dNTPs and 1.6 µl H_2O per sample. 16 µl of this Master Mix and 4 µl of the diluted DNA were mixed in the PCR tubes in triplets.

For the non-VCN gene, the same method was applied, using a modified Master Mix consisting of 10 µl GoTaq® qPCR Master Mix (Promega), the primers c_myc F (5'- GAG GGC CAA GTT GGA CAG TG -3') and CMYC_E3_R1 (5'- GGG CCT TTT CAT TGT TTT CCA -3') in a final concentration of 0.3 µM, and 4.8 µl H^2O. In each sample tube, 16 µl of Master Mix and 4 µl of the extracted and diluted DNA were mixed.

All reactions were run in triplicates and the first triplet contained a negative control consisting of 16 µl Master Mix and 4 µl H_2O, the second triplet contained our DNA standard sample, consisting of an equimolar mix of tissue extracts of sample number 6 which was used to construct the relative measure for all test samples..

The qPCR cycling conditions were an initial hold step at 95°C for 2 minutes to activate the polymerase followed by 45 cycles: 10 seconds at 95°C, 20 seconds at 59°C and 72°C for 20 seconds for the non-VCN gene. For the telomeres, cycling conditions were as for the non-VCN gene except annealing was performed at 56°C instead of 59°C. Telomere assay was run for 35

cycles. For all qPCR runs a melt step was included after the cycling where the temperature was stepwise increased by 1°C from 72 to 95°C. After each step the signal was acquired.

Table 1: PCR test runs for non-VCN gene: primer

	T 1	***T 2***
Promega	10 µl	10 µl
Forward Primer (10µM)	c-myc - F 0.6 µl	c-myc - F 0.6 µl
Reverse Primer (10µM)	c-myc - R 0.6 µl	**c-mycE3 - R 0.6 µl**
H²O	4.8 µl	4.8 µl
Step 1	10 sec 95°C	10 sec 95°C
Step 2	20 sec 61°C	**20 sec 59°C**
Step 3	20 sec 72°C	20 sec 72°C
efficiency	**1.794**	**1.897**

Table 2: PCR test runs for telomeres

	T1	***T2***	***T3***	***T4***	***T5***	***T6***
Promega	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Tel1b (10µM)	0.6 µl	0.6 µl	0.6 µl	0.6 µl	**1 µl**	**1.2 µl**
Tel2b (10µM)	0.6 µl	0.6 µl	0.6 µl	0.6 µl	**1 µl**	**1.2 µl**
dNTPs (2mM)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
H²O	2.8 µl	2.8 µl	**2.5 µl**	**2 µl**	**2 µl**	**1.6 µl**
BSA (50mg/ml)			**0.3 µl**			
MgCl² (25mM)				**0.8 µl**		
Step 1	10 sec 95°C	10 sec 95°C	10 sec 95°C	10 sec 95°C	10 sec 95°C	10 sec 95°C
Step 2	20 sec 56°C	**20 sec 58°C**	20 sec 56°C	20 sec 56°C	20 sec 56°C	20 sec 56°C
Step 3	20 sec 72°C	20 sec 72°C	20 sec 72°C	20 sec 72°C	20 sec 72°C	20 sec 72°C

efficiency	**1.729**	**1.729**	**1.678**	**1.697**	**1.840**	**1.805**

3.2.3 Statistical Methods

Relative telomere length (RTL) was calculated from non-baseline corrected raw data using LinRegPCR software. Calculation incorporated the Ct values and PCR efficiencies from telomere and non-VCN gene PCRs using the formula specified by (Ruijter et al. 2009) (Fig.1). For comparing RTL of the different tissue types and testing for significant differences, an analysis of variance (ANOVA) and Tukey multiple comparison of means (Tukey Test) were done, using RStudio. A Pearson and Spearman Test was applied to verify correlations between tissue types. This analysis helps us determine if the telomere dynamics in different tissue types behave in a similar way among individuals.

$$RTL = \frac{E_T^{\,Ct_T} / E_C^{\,Ct_C}}{E_{ST}^{\,Ct_{ST}} / E_{SC}^{\,Ct_{SC}}}$$

Fig.2: RTL = relative telomere length, E = qPCR efficiency, Ct = cycle threshold, T = telomere reaction of target sample, ST = telomere reaction of standard sample, C = control gene (c-myc) reaction of target sample and SC = control gene reaction of standard sample. (Hoelzl, Smith et al. 2016)

3.2.4 TRAP Assay

For detecting telomerase activity, a standard telomerase repeated amplification protocol (TRAP) was used, consisting of “three steps: extension, amplification and detection” (Mender and Shay 2015), in the case of this study using ddPCR, described in 3.2.4. .

For the cell lysate, a small piece (~10 mg) of tissue was put into a tube containing a cold metal bead without thawing and stored again on dry ice after pulverizing the tissue using a TissueLyser LT (Qiagen, Germany). The tissue powder was diluted with 40 µl of a cell lysis buffer (Ludlow et al. 2014), containing TrisHCl (pH8) in a final concentration of 10 mM, 1 mM $MgCl_2$, 1 mM EDTA (pH8), 1% (vol/vol) NP40, 25 mM SDC, 10% (vol/vol) glycerol, 150 mM NaCl, 5 mM BME and AEBSF in the concentration of 0.1 mM, and incubated on ice for 1 hour.

After incubation, 30 µl of cell lysate were saved for cell quantification and stored at -20°C and 1 µl of the cell lysate was transferred into a PCR-tube containing 50 µl of TRAP reaction MasterMix. The TRAP-MasterMix consisted of 38.6 µl H^2O, 5 µl of 10x TRAP buffer (200 mM Tris-HCl (pH 8.3), 15 mM MgCl2, 630 mM KCl, 0.5% Tween 20, 10 mM EGTA.), 0.4 µl BSA (50 mg/ml), TS primer (5'- AAT CCG TCG AGC AGA GTT -3') in a final concentration of 0.2 µM and 200 µM dNTPs. A HeLa cell lysate as positive control sample and a lysate negative sample were included in every TRAP run. The elongation conditions were 40 minutes at 25°C, followed by 5 minutes heat inactivation of the telomerase at 95°C. The TRAP reactions were than kept at 4°C until the upcoming ddPCR.

For the telomerase assay, multiple test runs were performed to optimise the assay, using just a few individuals and tissue types and also samples of a related species, garden dormice *(Tab.3)*. In some test runs, a 10 % chaps stock solution, consisting of 0.1 g chaps powder and 900 g sterile H^2O instead of NP40 was trialled.

3.2.5 Droplet Digital PCR

To detect and quantify telomerase activity, droplet digital PCR (ddPCR) was used whereby the PCR reaction mix is divided into thousands of droplets (in an oil matrix) before performing PCR and allows an absolute quantification of copy numbers (Gürtler and Gerdes 2014).

The ddPCR Master Mix consisted of 10 µl Eva Green Supermix (BioRad), TS primer and ACX primer (5'- GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3'), both in a final

concentration of 0.05 µM. The mix was toped up with H_2O to a final volume of 18 µl per reaction. This Master Mix and 2 µl Trap were mixed and transferred into the sample well of the droplet generator cartridge. After adding 60 µl Eva Green droplet oil to the oil well and running the droplet generating process, the droplets were slowly and carefully transferred to the PCR plate. A lysis, a TRAP negative and a PCR negative sample were included in each run. The ddPCR conditions were as follows: A hold step 5 minutes at 95°C, followed by 40 cycles at 30 seconds at 95°C, 30 seconds at 54°C and 30 seconds at 72°C.After the cycling the reactions were cooled for 5 minutes at 4°C and reheated for 5 minutes at 90°C. These steps were included to stabilize the droplets. The PCRs were kept at 12°C until read at the droplet reader.

Quantification of the input material (cell count) was not performed, as there was no telomerase activity detected for the different tissue types of edible dormice.

Tab.3: TRAP and ddPCR test runs on edible dormice samples

	T1	*T2*	*T3*	*T4*	*T5*	*T6*	*T7*
sample	All tissues 2 µl 1 µl	All tissues 2 µl	2 differently big pieces per tissue	MU_1_2 LE_1_2	MU_1_2	MU_1_2	LE_1 NI_1 MU_1
Dilution (amount of lysis buffer used)	100 µl	100 µl	100 µl	40 µl	100 µl	40 µl 100 µl metal bead removed	100 µl
detergens	NP40	NP40	CHAPS	NP40 and CHAPS in comparison	NP40	NP40	CHAPS and NP40
elongation	1 h	1 h	1 h	1 h	1 h	40 min 3h	1h

						9h	
ddPCR temperature	54°C	54 °C	54°C	54°C	Gradient 47.2°C - 61.3° C	54°C	54°C

4 RESULTS

4.1 Relative Telomere Length

All qPCR runs showed single peaks in the melt curve (*Fig.3)* and the negative sample was not amplified *(Fig.4).* The mean efficiencies of non-VCN runs ranged from 1,899 and 2.020, efficiencies of telomere runs were between 1,786 and 1,932.

The analysis of variance (ANOVA) showed a p-value $p < 0.001$, which means that there are significant differences between the tissue types *(Fig.5a, Fig.8a)*. A multiple comparison of all tissue types (Tukey test) showed that blood had significantly longer telomeres than all others ($p < 0{,}0001$) and muscle had significantly longer telomeres compared to mucosa ($p = 0{,}0177$) *(Fig.5b, Fig.6, Fig.7).* After removing blood from the data set, muscle tissue showed significant differences in RTL compared to liver ($p = 0{,}0092$), mucosa ($p < 0{,}0001$) and skin ($p = 0{,}0057$) *(Fig.8b, Fig.9).* RTL in brain samples was also significantly longer than in mucosa ($p = 0{,}0073$) *(Fig.8b, Fig.9).* The use of logarithmic values for the calculation had no relevant influence on the variance of the results.

The Pearson and Spearman correlation test showed a correlation of RTL between muscle and mucosa ($p = 0.0055$) and between liver and mucosa ($p = 0.0350$) (*Fig.10).*

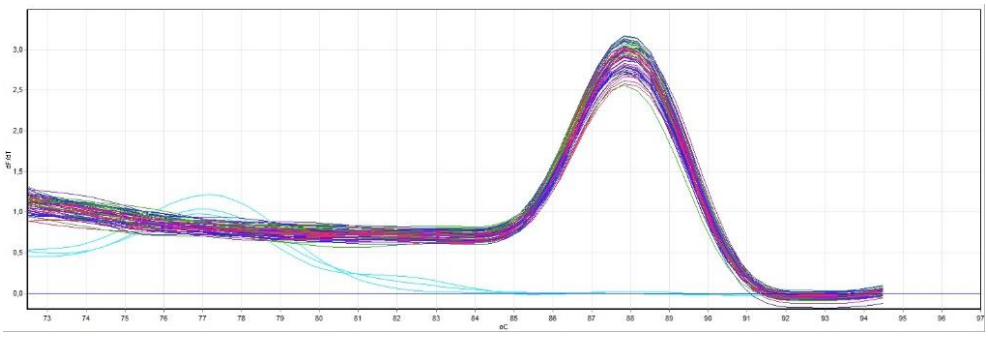

Fig.3: qPCR melt curve using liver tissue as example

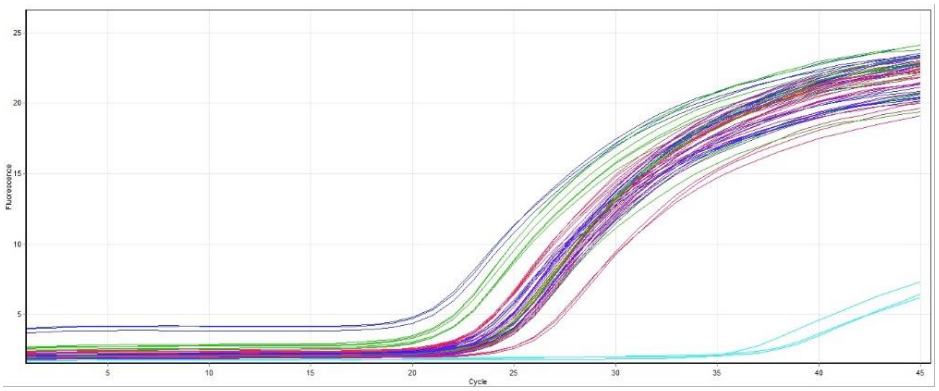

Fig.4: qPCR amplification using liver tissue as example

(light blue graphs = negative sample)

a)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
dat2$TissueT	5	149.9	29.972	65	<2e-16	*** *
Residuals	108	49.8	0.461			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

b)

$`dat2$TissueT`

	diff	lwr	upr	p adj	
GE-B	-2.78083667	-3.41204831	-2.1496250	0.0000000	*
LE-B	-3.05130487	-3.67437149	-2.4282382	0.0000000	*
M-B	-3.27539365	-3.89846027	-2.6523270	0.0000000	*
MU-B	-2.57357257	-3.19663919	-1.9505059	0.0000000	*
O-B	-3.11302272	-3.78601160	-2.4400339	0.0000000	*
LE-GE	-0.27046819	-0.90167983	0.3607434	0.8144908	
M-GE	-0.49455698	-1.12576861	0.1366547	0.2140846	
MU-GE	0.20726411	-0.42394753	0.8384757	0.9315403	
O-GE	-0.33218605	-1.01272270	0.3483506	0.7171106	
M-LE	-0.22408878	-0.84715541	0.3989778	0.9020303	
MU-LE	0.47773230	-0.14533432	1.1007989	0.2351362	
O-LE	-0.06171786	-0.73470673	0.6112710	0.9998145	
MU-M	0.70182108	0.07875446	1.3248877	0.0177308	*
O-M	0.16237092	-0.51061795	0.8353598	0.9815689	
O-MU	-0.53945016	-1.21243903	0.1335387	0.1928784	

Fig.5:

a) ANOVA (“df” =degrees of freedom, “sum sq” = sums of squares, “mean sq” = mean squares, “F-value”= result of F test, “Pr(>F)” = p value; p-values lower/equal p=0.05 are indicated with “”)*

b) Tukey multiple comparisons of means (‘diff’ = difference in means, ‘lwr’ and ‘upr’ = lower and upper limit of confidence interval, ‘p adj’ = adjusted p- values; p-values lower/equal p=0.05 are indicated with “”)*

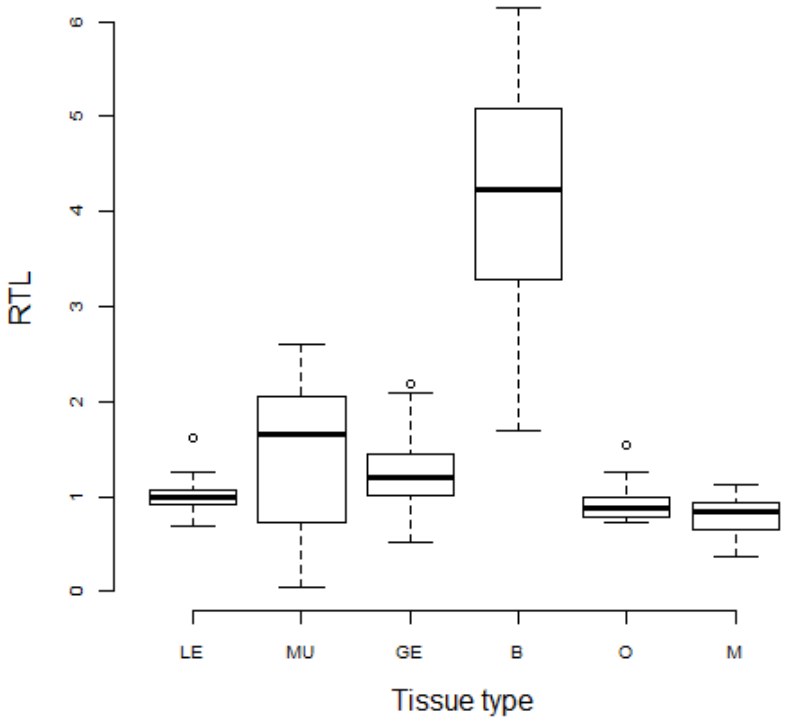

Fig.6: RTL of different tissue types

(LE = liver, MU= muscle, GE= brain, B = blood, O = skin, M = mucosa; n=114)

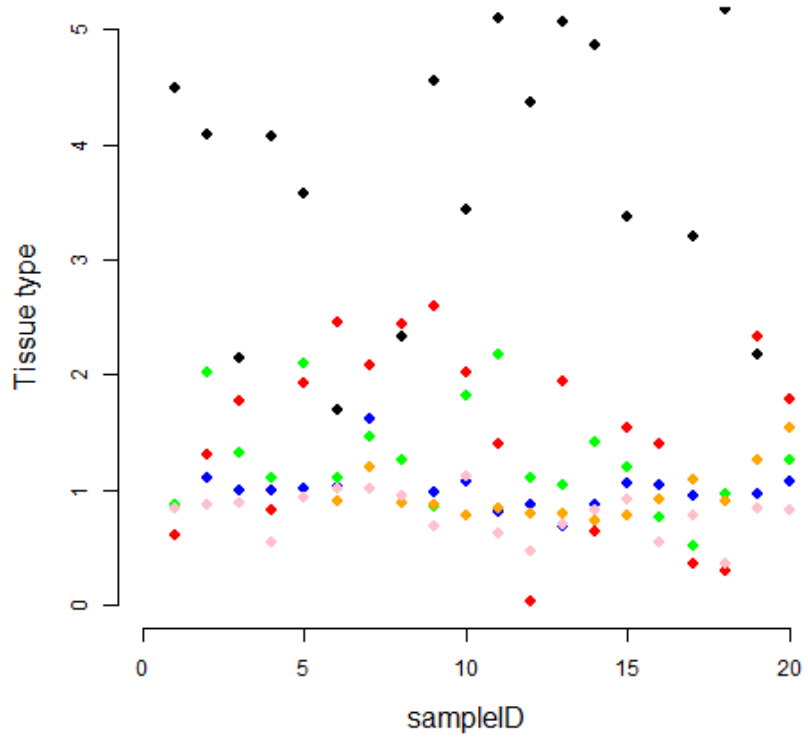

Fig.7: RTL per individual and tissue type

(blue=liver, red=muscle, green=brain, black=blood, orange=skin, pink=mucosa; n=114)

a)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
dat3$TissueT	4	6.099	1.5246	7.641	2.44e-05	***
Residuals	89	17.758	0.1995			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

b)

	diff	lwr	upr	p adj	
LE-GE	-0.27046819	-0.66892769	0.12799131	0.3302137	
M-GE	-0.49455698	-0.89301648	-0.09609748	0.0073426	*
MU-GE	0.20726411	-0.19119539	0.60572361	0.5983606	
O-GE	-0.33218605	-0.76178253	0.09741042	0.2073510	
M-LE	-0.22408878	-0.61740665	0.16922909	0.5099596	
MU-LE	0.47773230	0.08441443	0.87105017	0.0092331	*
O-LE	-0.06171786	-0.48654971	0.36311400	0.9942695	
MU-M	0.70182108	0.30850321	1.09513895	0.0000315	*
O-M	0.16237092	-0.26246093	0.58720278	0.8242369	
O-MU	-0.53945016	-0.96428201	-0.11461830	0.0057096	*

Fig.8:

a) ANOVA (“df” =degrees of freedom, “sum sq” = sums of squares, “mean sq” = mean squares, “F-value”= result of F test, “Pr(>F)” = p value; p-values lower/equal

p=0.05 are indicated with "")*

b) Tukey multiple comparisons of means ('diff' = difference in means, 'lwr' and 'upr' = lower and upper limit of confidence interval, 'p adj' = adjusted p- values; p-values lower/equal p=0.05 are indicated with "")*

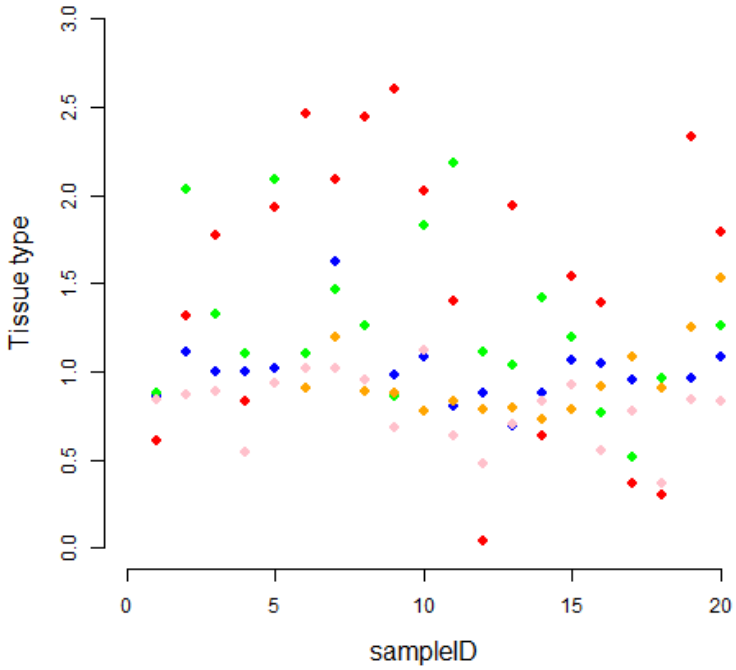

Fig.9: RTL per individual and tissue type without blood samples (blue=liver, red=muscle, green=brain, orange=skin, pink=mucosa; n=94)

	LE	MU	GE	B	O	M
LE	1.00	0.37	0.15	0.04	0.39	0.47*
MU	0.37	1.00	0.26	-0.30	0.21	0.60*
GE	0.1	0.26	1.00	0.00	-0.14	0.37
B	0.04	-0.30	0.00	1.00	0.19	-0.42
O	0.39	0.21	-0.14	0.19	1.00	0.15
M	0.47*	0.60*	0.37	-0.42	0.15	1.00

Fig.10: correlation coefficient R

(p - values lower/equal to p=0.05 are indicated with "")*

4.2 Telomerase Activity

There was no telomerase activity detected with the ddTRAP method in any of the tissue samples used in this assay. As including of HeLa cells as a positive control sample and the use of garden dormice tissue samples from different sampling times gave positive results, we can rule out the possibility of a technical reason for the failed assay.

5 DISCUSSION

5.1 Relative Telomere Length

We found that RTL varies across different tissue types. In almost all individuals (17/20), blood cells had the longest telomeres. As erythrocytes and thrombocytes of mammals have no cell nucleus and therefore no DNA, only leukocytes can show those long telomeres and reflect RTL of hematopoietic stem cells in the bone marrow (Daniali et al.).
Leukocytes include many specific types of cells, such as granulocytes, monocytes and different kinds of lymphocytes, all of them responsible for different stages of immune defence.
Working leukocytes are very important for organism health, as they need to proliferate for fighting infections and maintaining an immunological commemoration (Aubert and Lansdorp 2008). It is furthermore assumed, that oxidative stress often has a negative impact on the immune system and accelerates aging of these cells, whereby infections encourage the development of free oxygen radicals – a sort of vicious cycle.
Compared to other tissues cells, leukocytes are short living and have a quick turnover rate depending on their function, from 20 to 60 days (Trepel and Rastetter 1967). Furthermore, long telomeres ensure many cell divisions which are essential for immune reactions and the following maintenance of memory cells (Weng 2008).
Thus, long telomeres help to prevent aging as well as related diseases and mutations of these cells caused by frequent divisions. Mutations in progenitor cells of leukocytes, leading to leukaemia (Pui et al. 2008), would have disastrous impact due to spreading in all organs apart from the impact of non-threatened pathogens.

Apart from blood, muscle showed significantly longer telomeres compared to buccal mucosa, skin and liver. In our test, we used skeletal muscle, an example of striated muscle. Skeletal muscle is responsible for motion sequences and postures but also for energy production by reducing glycogen (anaerobic energy production) or by reducing glycogen, lactate and fat acids (aerobic energy production) and subsequently producing ATP. Aside from that, muscle serves as a repository for amino acids for other organs, like liver and brain. (Engelhardt et al. 2015).

It is established that myoblasts (satellite cells), stem cells of muscle tissue, are essential for renewing and regeneration (Hawke and Garry 2001). As the muscle fibres, consisting of myofibrils and a number of nuclei, are not able to divide, satellite cells make new cells available in cases of hypertrophy, due to growth or training, or myotrauma (Hawke and Garry 2001).
With aging or chronic diseases, the function and amount of satellite cells decreases and causes a loss of muscle mass (Muñoz-Cánoves et al. 2019). Furthermore, oxidative stress in muscle tissue is thought to affect cachexia (Ábrigo et al. 2018).
As muscle mass changes over a lifetime and only satellite cells are able to renew muscle cells, long telomeres would ensure production of healthy muscle cells and furthermore the maintenance of energy production.
In a similar study by Reichert et al. 2013, telomere length in tissues of birds was measured and they found muscle having the longest telomeres that significantly differ from liver, followed by red blood cells, which are owning a nucleus and DNA in birds and reptiles, whose RTL was significantly different to spleen. Even if the differences in RTL are not as clear as in our study, the same tissues show longer telomeres in comparison to others.

Brain cells showed significantly longer telomeres than mucosa. The most important brain cells are neurons, which are surrounded by different types of glia cells. In contrast to other cells, neurons do not replicate and, therefore, the length of telomeres do not have a great impact with respect to aging (Engelhardt et al. 2015). Even though it was thought for a long time that brain cells are not able to regenerate at all after birth, it is known now, that new neurons are generated in the dentate gyrus for a lifetime, which are synaptically connected to the hippocampus (Cameron and Glover 2014).
Glia cells are supporting cells that isolate neurons by building myelin sheets and contribute to synaptogenesis, apart from many other metabolic and regulating functions. As they are able to divide and reach senescence, which can cause many neuronal diseases like multiple sclerosis or Alzheimer's disease, longer telomeres would be beneficial due to their importance in maintenance of the nervous system. (Zuchero and Barres 2015)
Oxidative stress is known as a causal agent of neurodegenerative diseases (Chen et al. 2012) against which telomeres could provide a protective function.

Liver, skin and mucosa cells seem to have telomeres of similar length.

The most superficial layer of skin, the epidermis, which is cornified, shows a high turnover as its stratum basale is responsible for renewing of the epithelial cells (Wong et al. 2016). Collagen in the other layers, dermis and hypodermis, ensures flexibility and stretching. A loss of thickness of the epidermis and of collagen in the other layers cause wrinkles and less elastic skin in higher ages. (Wong et al. 2016)

As epidermis gets thinner, it would also mean that there are less cells and a decrease in cell turnover when the individuum is aging. Furthermore, skin cells are affected by oxidative stress due to environmental impacts that accelerate aging and often causes cancerous mutations (Godic et al. 2014). The epithelial layer of mucosa is, in contrast to skin, not cornified and the submucosal layer contains various glands (Liebich and Budras 2010). In contrast to skin, the epithelial layer of mucosa is not thinning with age (Stablein and Meyer 1988) but the cells tend to change their size and shape (Abu Eid et al. 2012). Furthermore, mucosa is not exposed to environmental impacts like UV radiation, which has a hugh effect on aging of skin (Krutmann 2003) but through food intake and chewing, oral mucosa is exposed to a lot of irritation and trauma, which leads to chronic inflammation and oxidative stress (Singhvi et al. 2017).

Liver cells consist of many different cell types with a variety of functions. The epithelial cells differentiate from hepatoblasts, whereby hepatocytes build the lobes and cholangiocytes have an epithelial function. Stellate cells store Vitamin A and radiate it in cases of damage, as well as deposing collagen which causes scars. Kupffer cells are macrophages and also endothelial cells are present. Due to partitioning in different zones, liver is able to replace loss of function in one zone by another one. (Trefts et al. 2017)

Hepatocytes do not divide without stimulation by injuries and, with aging, they change their structure and the volume of liver decreases. Most degenerative liver diseases are also age-related. (Gregg et al. 2012)

Thus, the common factor for all these tissue types is that they are changing in cell count or volume with increasing age. Furthermore, skin and mucosa are exposed to high levels of oxidative stress. Another reason for the short telomeres in these tissues could be the finite resources to elongate them. It is possible that telomerase could be too costly to produce into autumn, when the samples were taken.

Interestingly, we could detect a correlation between RTL of liver and mucosa, and muscle and mucosa. This means, that individuals with longer telomeres in liver tissue also have longer ones in mucosa cells, and vice versa. Liver and mucosa show similarities in the length of telomeres, so it could be that those cells have analogies in division and replication behaviour. As muscle and mucosa are very different in telomere length and also in occurrence of mitosis, a correlation is somewhat surprising. It seems like there are interindividual differences in the way tissues respond in telomere length, which is surprising since all individuals were kept in the same way. Of course, a stochastic correlation created by the sample range cannot be excluded.
In a similar study by Reichert et al. 2013 strong correlations of blood cells with other tissues were measured whereby one could assume blood as a representative sample for the entire organism. However, one has to keep in mind that leukocytes in mammals may show different processes and that the age group also has to be considered. (Reichert et al. 2013).

What can be excluded, though, are errors introduced into our results produced by unevenly sized pieces of sample tissue we took for extracting DNA, as we normalised the concentration of DNA for the measuring steps and standardised the measurements against a non-variable copy number gene. Also, big differences in RTL caused by age-related shortening of telomeres can be neglected because only samples of yearlings were used for these measurements.

5.2 Telomerase Activity

One reason for why we were not able to detect any telomerase activity in the samples of edible dormice tissues could be the time of the sampling. The yearlings were killed in autumn and as explained above, edible dormice elongate their telomeres in summer. Therefore, it could be, that telomerase is not expressed or just inactive in autumn when the animals have to finally prepare for the upcoming hibernation season.
Another reason for not detecting telomerase activity could be a negligence in taking and preserving the samples. Telomerase is very sensitive to warmth and so, the samples should be frozen immediately after taking. As the samples were taking within minutes after sacrificing the animals and were flash frozen and stored at -80°C we can rule out this reason for not detecting any telomerase activity, as the same procedure was used for the garden dormice

samples (taken at the same time for a related experiment and which gave positive signals). Furthermore, the samples were always kept on dry ice when processed in the lab.

6 CONCLUSION

After testing 20 individuals of edible dormice it is clear that there are consistent differences in telomere length across different tissue types. Blood cells showed the longest telomeres compared to other tissue types and could be explained by the high need of cell renewal due to the short lifespan of leukocytes and their high turnover rate, which accelerates mutations, as well as the importance of maintenance of a healthy immune system to prevent diseases partially provoked by oxidative stress. The second longest telomeres where documented in muscle cells, more specifically in division active satellite cells, whose importance lies in adaption to muscle growth or healing of trauma, an indispensability for the perpetuation of metabolism, motion and energy production. RTL in brain cells was significant longer compared to mucosa cells and can perhaps be traced to glia cells and their importance regarding the prevention of neuronal diseases as neuronal cells show no turnover. Liver, mucosa and skin showed the shortest telomeres and these tissues could be expected to show a loss of cells or change in tissue volume and thereby a related decrease in cell turnover with aging. One explanation could be the high exposure to oxidative stress, another one could be the preference to elongate other important cell types first and the exhaustion of telomerase at the end of summer, when our samples were taken.
Mucosa showed a correlation with liver and muscle cells.

Since all individuals tested were the same age, measured differences in RTL can be attributed to the tissue type and not affected by age related shortening. By adjusting the concentration of DNA before measuring RTL we can also exclude error caused by sample pieces of different sizes. It would be interesting to also test, how the RTL changes with age and whether the relations remain the same, as well as whether tendencies of specific tissue cells to shorten their telomeres faster can be shown.

Telomerase activity could not be detected in any samples of edible dormice. One reason could be the time at which the samples were taken. As edible dormice elongate their telomeres over summer, telomerase could be depleted in autumn, when our sampling took place. It is therefore interesting to take new samples throughout the year, particularly in May or June when they start

to elongate their telomeres after hibernation. After that, one could test again the correlation between telomerase activity and telomere length in all tissue types.

Research in telomeres and also telomerase activity is of great importance and interest, since aging, age-related reduction and unfortunately also cancer affects almost every species. Knowing the genetic basis behind those diseases could uncover prophylactic options and can make an important step to further development of cancer treatment.

7 SUMMARY

The aim of this study was to find out if the length of telomeres, short sequences of DNA that cap our chromosomes and protect them from shortening due to the end replication problem, varies across different tissue types of edible dormice. For that, samples of 20 yearlings were taken, DNA was extracted and the relative telomere length of brain, liver, mucosa, skin, blood and muscle was calculated using quantitative PCR. We found that blood shows significant longer telomeres compared to the other tissues, which can perhaps be explained by their short lifespan and the need to divide and renew from hematopoietic stem cells in bone marrow very often to fight infections and provide a healthy immune system. Muscle showed significant longer telomeres and after removing blood from the comparison, also brain compared to mucosa. Satellite cells, the only dividable cells of muscle tissue, are essential for renewal after trauma and muscle growth. Mutations, benefited by short telomeres, in these cells would affect metabolism, motion and energy generation. Healthy glia cells are essential for the prevention of neurodegenerative diseases. The short telomeres of liver, mucosa and skin could be explained by volume and cell loss of the tissues in higher ages, the high exposure to oxidative stress or the preference to elongate other tissues first. These conclusions should be treated with caution however as the correlation between muscle, liver and mucosa could be an artefact of stochastic chance and further replication of this study is recommended.

In the context of telomere length, we also wanted to show the activity of telomerase in these tissues and if it correlates with the RTL. To detect the enzyme, which is responsible for the elongation of telomeres that occurs for example in older edible dormice and after their hibernation season, we used ddTRAP assay to gain DNA, amplify it and then detect telomerase using ddPCR. In none of the test runs on edible dormice samples could telomerase be detected. While this could be ascribed to errors during sampling the more likely explanation is that by the time of sampling (autumn), after elongation in summer, all telomerase could be depleted. To continue research, new samples should be taken, preferably in early summer when edible dormice start to elongate their telomeres.

8 ZUSAMMENFASSUNG

Das Ziel dieser Studie war, herauszufinden, ob Telomere, kurze DNA-Sequenzen am Ende der Chromosomen, die das Genmaterial bei jeder Zellteilung auf Grund des Endreplikationsproblems vor Verlusten schützen, in verschiedenen Gewebezellen des Siebenschläfers unterschiedlich lang sind. Deswegen wurden Proben von 20 Jährlingen genommen, DNA extrahiert und die Telomerlänge von Blut, Muskel, Gehirn, Leber, Mukosa und Haut mit der quantitativen PCR bestimmt. Es stellte sich heraus, dass Blut verglichen mit den anderen Geweben signifikant längere Telomere aufweist. Da Leukozyten eine kurze Lebensdauer haben und sich, um Infektionen bekämpfen zu können, oft erneuern und teilen müssen, sind lange Telomere Voraussetzung für die Aufrechterhaltung eines gesunden Immunsystems. Muskelgewebe hatte signifikant längere Telomere, nach Vernachlässigung von Blut auch Gehirn verglichen mit Mukosa. Satellitenzellen sind als die einzigen teilungsfähigen Zellen des Muskels zuständig für die Anpassung an Wachstum und Gewebserneuerung nach Traumata. Mutationen in diesen Zellen hätten Auswirkungen auf Metabolismus, Körperhaltung und Energiegewinnung. Gesunde Gliazellen des Gehirns sind unerlässlich für die Prävention von neuronalen Erkrankungen. Die kurzen Telomere in Leber-, Mukosa- und Hautgewebe könnten durch den Gewebsverlust im Alter, die hohe Exposition zu oxidativem Stress oder das bevorzugte Investieren von Telomerase in wichtigere Gewebe erklärt werden. Diese Rückschlüsse sollten mit Vorsicht betrachtet werden, so wie auch die Korrelation zwischen Mukosa und Muskel sowie Leber ein Zufallsergebnis sein könnte - eine Replikation der Studie wäre empfohlen.

Im Zusammenhang mit der Telomerlänge war die Aktivität des Enzyms Telomerase, durch welches der Siebenschläfer seine Telomere im Alter und nach dem Erwachen aus dem Winterschlaf verlängert, von Interesse. Zur Messung des Enzyms wurde ein ddTRAP Assay durchgeführt, um DNA zu gewinnen, zu amplifizieren und danach mit Hilfe der ddPCR das Enzym zu quantifizieren. In keinem der Durchläufe konnte Telomerase detektiert werden, was entweder auf einen Fehler bei der Probennahme oder auf den Zeitpunkt der Probennahme, nämlich im Herbst am Ende der Verlängerungsperiode, an dem die Telomerase aufgebraucht sein könnte, zurückzuführen ist. Zur weiteren Forschung sollten neue Proben im Sommer entnommen werden.

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10 TABLES AND FIGURES

Figure 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: Clara Noé-Nordberg

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11 GLOSSARY

DNA deoxyribonucleic acid

RNA ribonucleic acid

dNTPs deoxyribonucleotide triphosphates

TRF terminal restriction fragmentation

PCR polymerase chain reaction

qPCR quantitative polymerase chain reaction

MMqPCR monochrome multiplex quantitative polymerase chain reaction

aTL absolute telomere length

STELA single telomere length analysis

Q-FISH quantitative fluorescent in situ hybridisation

NaCl Natriumchlorid = sodium chloride

TrisHCl Tris(hydroxymethyl)aminomethan

SDS sodium dodecyl sulfate

EDTA ethylendiamintetraacetate

$MgCl^2$ magnesium chloride

NP40 nonylphenolethoxylate

SDC sodiumdesoxycholate

BME betamercaptoethanole

AEBSF aminoethylbenzensulfonylfluoride

BSA bovine serum albumine