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Characterisation of telomere length dynamics in dairy cattle and association with productive lifespan

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Abstract

Telomeres form protective caps at the ends of linear chromosomes. They consist of repetitive DNA nucleotides and associated proteins of the shelterin complex. In vitro telomeres become shorter during cell division and when a critical shortness is reached they trigger a DNA damage response that leads to replicative senescence or apoptosis. Telomere shortening is a recognised hallmark of cellular ageing and seems to be also associated with organismal ageing. Telomere length (TL) and the rate of shortening vary across individuals and several studies have found that short telomeres and fast telomere depletion are associated with poor survival and early onset of age related diseases. However, longitudinal studies are needed to better understand the relationship of TL and TL dynamics with longevity measures. Relevant studies on livestock species are largely missing from the literature.

In the dairy industry, farmers are forced to cull a considerable percentage of their heifers and cows at a young age due to fertility problems or diseases. As a consequence many replacement heifers have to be reared to maintain a specific herd size. This results in increased costs, consumption of resources, and damage to the environment. Breeding for an improved productive lifespan is difficult because longevity measures are recorded at the end of life and are known to have a low heritability. Therefore, the expected genetic improvement is generally slow, but could be considerably accelerated if an early life heritable biomarker was identified that is predictive of productive lifespan and could be used for animal selection. The question is if TL could be used as such a biomarker.

The objectives of this thesis were to 1) develop robust methods to measure average relative leukocyte TL (RLTL) in cattle, 2) examine RLTL dynamics with age at a population as well as at an individual level, 3) estimate genetic parameters and 4) assess the association of RLTL and RLTL dynamics with productive lifespan.

A quantitative polymerase chain reaction (qPCR) based assay developed for human studies was adapted to cattle and delivered robust results (repeatability > 80%, coefficient of variation=0.05). Different DNA extraction methods were tested for their effect on RLTL measurements and it was demonstrated that fast silica based DNA extraction methods are suitable for telomere projects which can improve the sample throughput and enable large-scale projects. Subsequently, RLTL in 1328 whole blood samples of 308 Holstein Friesian dairy cows and additionally in 284 whole

Abstract

blood samples of 38 female calves was measured. Repeatability and random regression models were used for the statistical analysis of telomere data.

RLTL decreased considerably within the first year of life, but remained relatively stable afterwards at population level. Animals varied significantly in their amount and direction of telomere change. The genetic correlation between consecutive measurements in the same individual weakened with increasing sample interval from $r=1$ to $r=0.69$ which indicates that TL in the beginning of life might be under a different genetic control than TL later in life. For the first time in a livestock species we calculated heritability estimates for RLTL which were high (0.32-0.38) and remained constant over life. Long telomeres at birth were not predictive of better productive lifespan. However, animals with long RLTL at the ages of one and five years had a survival advantage. Also, animals that showed less average RLTL attrition over their lives remained in production for longer.

TL dynamics differed among individuals and a considerable subset of individuals demonstrated telomere lengthening between consecutive measurements. On average, telomeres tend to shorten early in life and then remain relatively constant. While TL is a heritable trait throughout lifetime, telomere change is not heritable. Short TL at specific ages and telomere attrition over life were associated with poorer productive lifespan.

Lay summary

Lifespans vary considerably between individuals of the same or different species. The reasons for such differences are not completely understood. However, some molecular mechanisms that can cause ageing of cells and potentially variation in lifespan among individuals have been identified. One of these mechanisms is the shortening of the ends of chromosomes which are called telomeres.

Telomeres consist of DNA and proteins. They have many important functions and characteristics, one of which is the fact that they tend to shorten with age. In some studies it has been found that they are also associated with the lifespan of individuals: Long telomeres were linked to better survival.

For dairy cows the improvement of productive lifespan is a key breeding goal because it would save money and resources, benefit the environment and improve animal welfare. Fast genetic improvement can be expected for traits that are transmitted from one generation to the next and can be measured relatively early in an animal's life. While in the case of productive lifespan the first is partially true, the second is not the case; as a consequence the genetic improvement of productive lifespan is slow. It would be tremendously helpful to identify a measure that fulfils those requirements (being measurable early in life and caused by genes) and at the same time predicts productive lifespan. Such a measure could be used to accelerate the improvement of productive lifespan in the dairy cattle population considerably.

In this thesis, a robust technique for the measurement of average telomere length in white blood cells of dairy cattle is presented. Further, the change of telomere length with age is explored. The analysis is based on a total of 1612 samples taken from 346 female animals. Each animal was sampled shortly after birth and at least one more time later in life. Telomeres shorten within the first year of the animals' lives but, on average, remain stable afterwards. However, these trends differ among individuals.

Telomere length at all ages is influenced by the genetic makeup of the animal which means that telomere length is a trait that can be passed onto the next generation. However, telomere length early in life seems to be influenced by different genes than telomere length later in life. Telomere length at the ages of one and five years

Lay Summary

is strongly associated with subsequent survival; animals with long telomeres and also animals that shorten their telomeres less have longer productive lifespans.

This thesis contributes to a better understanding of telomere change over life in dairy cattle. It shows that telomere length is partially influenced by the animal's genes and that it may be associated with productive lifespan. If telomere length can be used as a tool in breeding schemes to improve the productive lifespan of dairy cattle requires more investigation and testing.

Acknowledgements

This telomere project started with a simple conversation between Mike Coffey and Bill Hill - I believe during a car ride. I am incredibly grateful for this conversation and for the expertise and creativity of my four wonderful supervisors Georgios Banos, Dan Nussey, Mike Coffey and Bruce Whitelaw to transform this idea into an interesting project that even attracted additional BBSRC funding for work that goes beyond the contents of this thesis.

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Declaration

I hereby declare that this thesis has been composed by me and that I made a substantial contribution to the work presented. My contribution is clearly indicated throughout the thesis. I also declare that the work has not been submitted for any other degree or professional qualification. My contributions to publications that are included in the thesis are clearly identified in the beginning of each chapter.

Luise Avelina Seeker

For my family.

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Abbreviations

%	Percent
° C	Grad Celsius
A	Adenine
AIC	Akaike information criterion
ALT	Alternative lengthening of telomeres
B2M	Beta-2-microglobulin
C	Cytosine
cov	Covariance
Cq	Cycle at which qPCR amplification curve crosses threshold
CV	Coefficient of variation
df	Degrees of freedom
D-loops	Displacement loops
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
E	qPCR efficiency
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence in situ hybridisation
G	Guanine
g	Gravitational force
gDNA	Genomic DNA
h ²	heritability
HF	High forage
ID	Identity
LF	Low forage
LogL	Log-likelihood
LogLratio	Log-likelihood ratio test
LTL	Leukocyte telomere length
mA	Milliampere
min	Minute(s)
ml	Millilitre
µl	Microliter
MS	Method specific
MS.cal	Method specific calibrator
mV	Millivolt
N	Total number
N.B.	Note bene (“observe carefully”)
NaCl	Sodium chloride
ng	Nanogramm
nm	Nanometre
nmol	Nanomol
No.cal	No calibrator
OD	Optic density
ORI	Origin of replication
p	p-value
PBS	Phosphate-buffered saline
PG	Puregene
PG.cal	Puregene calibrator
POT1	Protector Of Telomeres 1 (shelterin protein)
QC	Quality control

Q-FISH	Quantitative fluorescence in situ hybridisation
qPCR	Quantitative polymerase chain reaction
r	Correlation coefficient
ρ	Spearman's Rho statistic
r^2	Coefficient of determination
r_A	Genetic correlation
RAP1	Protein of the shelterin complex
RBC	Red blood cell
REML	Residual maximum likelihood
RLTL	Relative leukocyte telomere length
RNA	Ribonucleic acid
rpm	Rounds per minute
RTL	Relative telomere length
s	Second(s)
S	Select
S1/2	Supplementary 1/2
σ^2	Variance
SC	Spin column
SD	Standard deviation
sd	Standard deviation
SE	Standard error
SP	Spin plate
STELA	Single telomere length assay
T	thymine
t	t-statistic
TEL	Telomere
TERC	RNA template of the enzyme telomerase
TERT	Telomere reverse transcriptase (catalytic centre)
TIN2	TRF1 interacting protein 2 (shelterin protein)
TL	Telomere length
T-loops	Telomere loops
TPP1	Protein of the shelterin complex
TRF	Terminal restriction fragment
TRF1	Telomere Repeat Factor 1 (shelterin protein)
TRF2	Telomere Repeat Factor 2 (shelterin protein)
TZAP	Telomeric zinc finger associated protein
UK	United Kingdom
UV-light	Ultra violet light
V.	Vena
V_A	Additive genetic variance
var	Variance
V_P	Phenotypic variance
W	Shapiro Wilk statistic
X^2	Chi squared statistic

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Chapter 1

General introduction

1.1 Telomeres

1.1.1. Definition of telomeres

In 1938 Herman Muller coined the word “telomere” to describe the physiological ends of linear chromosome (Figure 1), in contrast to the term describing their middle part, the centromere (Muller 1938). The term is derived from the Greek words telos (τέλος = end) and meros (μέρος = part). Herman Muller and Barbara McClintock observed in the 1930s in fruit flies and maize that free ends of DNA emerging after chromosome breakage are “sticky” and lead to chromosome fusion while intact natural chromosome ends never induce such a cellular reaction (McClintock 1939; Muller 1938). They concluded that chromosome ends must be structurally different from DNA double strand breaks, even though it was impossible at that time to investigate their molecular structure (reviewed by Blackburn et al., 2006).

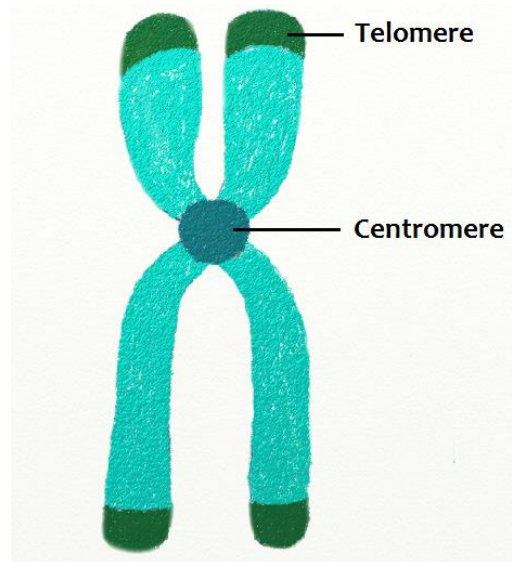


Figure 1: Eukaryotic linear chromosome.

1.1.2. Structure of telomeres

Telomeres consist of tandemly repeated DNA nucleotides which are bound by proteins. The nucleotide sequence of telomeric DNA was first decoded for the ciliate *Tetrahymena thermophila* in 1978 (Blackburn & Gall 1978). Elisabeth Blackburn observed that the same DNA sequence (CCCAA) was repeated 20 to 70 times at the ends of the ciliate’s mini chromosomes. A decade later, the telomeric DNA sequence “TTAGGG” was decoded for humans (Moyzis et al. 1988) and shown to be well conserved amongst vertebrate species (Meyne et al., 1989; Meyne et al., 1990). Today we know that across all eukaryote taxa the presence of repetitive DNA at chromosome ends is

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well conserved (Blackburn, 1991; Meyne et al., 1989; Witzany, 2008). The actual base sequence may vary although it is normally known to be guanine-rich (Blackburn, 1991; Meyne et al., 1989; Witzany, 2008). The length of telomeres is dynamic and varies between chromosomes, cells, tissues, individuals, and species (Blackburn, 1991; Friedrich et al., 2000; Gomes et al., 2011; Lin et al., 2010; Martens et al., 1998; Nasir et al., 2001). Telomeric DNA is mainly double stranded but the guanine-rich strand produces a 3' overhang (Blackburn, 1991).

Telomeric DNA is bound and protected by a complex of six different proteins which are collectively called the “shelterin complex” or “telosome”. In vertebrates shelterin proteins include the Telomere Repeat Factors 1 and 2 (TRF1 and TRF2, respectively) that directly bind double stranded telomeric DNA and the Protector of Telomeres 1 (POT1) that interacts with the single stranded telomeric 3' overhang. Other proteins including RAP1, TIN2 (TRF1 interacting protein 2) and TPP1 bind not directly to telomeric DNA but shelterin proteins and are important for the formation of the shelterin complex (De Lange, 2005; Palm & de Lange, 2008; Schmutz & De Lange, 2016; Xin et al., 2008).

1.1.3. Functions of telomeres

Telomeres form protective caps that prevent normal chromosome ends from being confused with DNA double strand breaks. Shelterin proteins facilitate the formation of telomere loops (T-loops) at the ultimate part of telomere structure: Telomeres fold back onto themselves and the single strand 3' overhang is tucked into the double stranded telomeric DNA that forms a displacement loop (D-loop) for this purpose (Greider, 1999; Griffith et al., 1999). Through the formation of T-loops, both the 5' and 3' ends of the telomere are hidden from the DNA repair machinery, which prevents end to end fusions of intact chromosomes. Therefore, T-loops contribute to the molecular distinction between normal chromosome ends and double strand breaks, as had been hypothesised by Barbara McClintock and Hermann Muller (McClintock 1939; Muller 1938). Uncapped chromosomes that lack functioning telomeres, on the other hand, trigger breakage fusion bridge cycles (McClintock, 1939; Murnane, 2013; Stewart et al., 2012). If several uncapped chromosomes are present within the same cell, their ends are fused together in an attempt to repair what is perceived as a DNA double strand break. This leads to the formation of dicentric chromosomes. During the anaphase of the next cell division both centromeres of the dicentric chromosome will be pulled to opposite cell poles and

the dicentric chromosome will form a bridge-like structure before it tears at a random site between its two centromeres. The ends of the teared chromosome are again not protected by telomeres and, therefore, the cycle starts anew in both daughter cells. If no other uncapped chromosomes are present within the same cell, it is likely that sister chromatids fuse together leading to excessive chromosomal rearrangements (Stewart et al. 2012; Murnane 2013). Such chromosomal abnormalities are likely to be fatal for the cell. Therefore, intact telomeres are crucial for the maintenance of a normal chromosomal structure and function.

Telomeres are also important for a correct chromosome segregation during meiosis, because they play a role in the pairing of homologous chromosomes (Ding et al., 2004; Niwa et al., 1996). This is not only important for the formation of intact germ cells but also promotes variation amongst offspring through homologous recombination.

As mentioned above telomere sequences vary across eukaryote taxa but are usually guanine-rich (Blackburn, 1991; Meyne et al., 1989; Witzany, 2008). This is important for the function of telomeres as a detector of DNA damage because guanine is particularly susceptible to DNA damaging influences such as UV-light and radiation (Henle et al. 1999). Telomeres have been shown to be more sensitive to DNA damage induced by X-ray irradiation, UV-light and oxidative stress than the rest of the chromosomes (Henle et al., 1999; Hewitt et al., 2012; Kruk et al., 1995; Oikawa et al., 2001). Even though telomeric DNA contributes only a fraction to the total amount of genomic DNA, 50 % of X-ray induced DNA damage response foci were found within telomeric sequence (Hewitt et al. 2012). Furthermore, telomeres not only seem to be more susceptible to DNA damage they also seem to be less efficient in repairing their damage and therefore more frequently show a persistent DNA damage response (Hewitt et al. 2012). Oxidative stress and ultra violet A irradiation accelerate telomere shortening (von Zglinicki 2002; Oikawa et al. 2001) and accumulated DNA damage within telomeres can lead to stress induced senescence. Stress induced senescence is probably a protection mechanism where the cell is removed from the replicative pool to shield the organism from the accumulation of DNA damage in coding or regulatory regions of its DNA (Hewitt et al. 2012). Because of this function telomeres have been compared to a “canary in the coal mine” (Blackburn et al., 2015).

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Telomeres have no gene-coding function. However, they can influence the transcription of coding genes which they probably do in a telomere length (TL) dependent manner: Long telomeres form bulky structures that inhibit the transcription of genes in their close proximity. When telomeres become shorter they uncurl and genes in their neighbourhood can be expressed more readily. This is known as telomere position effect (Baur et al., 2001; Robin et al., 2014). Telomeres have also been shown to loop to genes that are further away in order to modulate their expression. This process is known as telomere position effect over long distance (Kim et al., 2016; Robin et al., 2014). Shelterin proteins can also alter the expression of genes by acting as transcription factors (reviewed by Blackburn et al., 2015).

The enzymes that copy eukaryotic DNA in preparation for cell division, mainly DNA polymerases alpha and delta (Tsurimoto & Stillman 1989), only work in the 5' to 3' direction. Therefore, DNA replication is continuous on the leading strand, but discontinuous on the lagging strand (Okazaki et al., 1968). As a consequence of the discontinuous DNA synthesis on the lagging strand and the inability of DNA polymerases to work in the 3' to 5' direction, the ultimate primer on the 3' end of the template DNA cannot be replaced and a few DNA bases (approximately 10-20 base pairs in human fibroblasts (von Zglinicki 2002)) are lost with every cell division. This is termed the "end replication problem" (Watson 1972; Olovnikov 1973) (Figure 2).

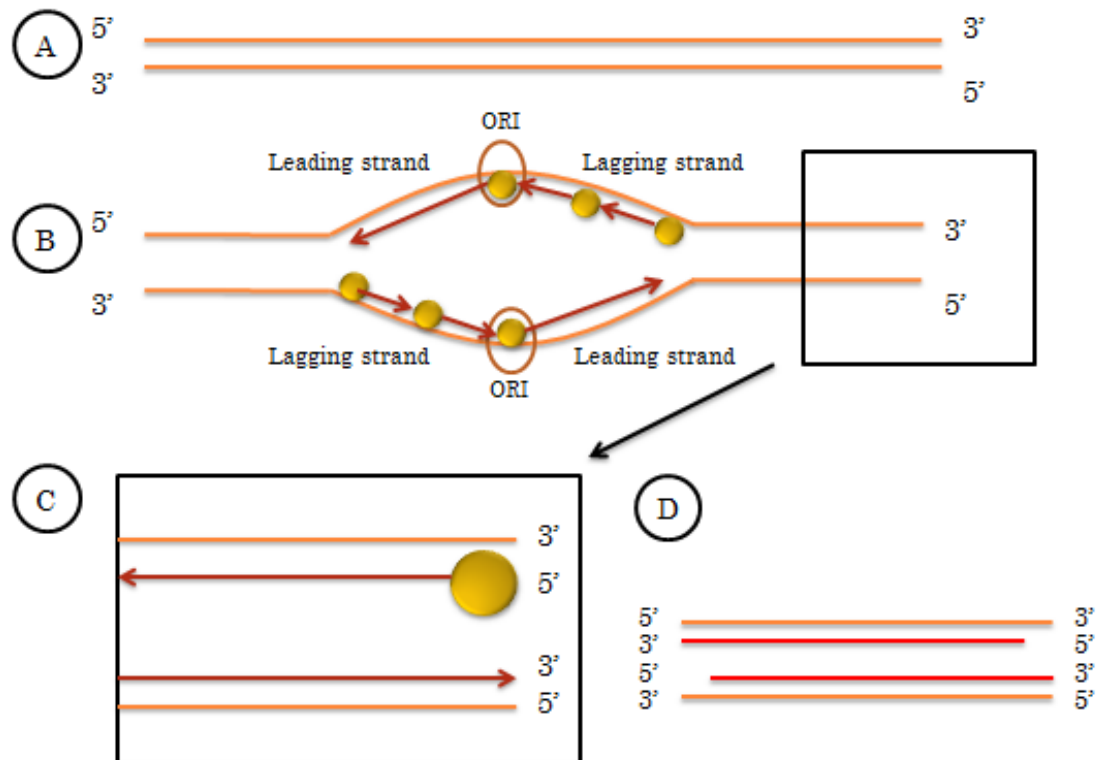


Figure 2: The “end replication problem”. (A) DNA strand before replication. (B) Origin of replication (ORI) as yellow dots. Red arrows represent newly synthesised DNA strand. On the Leading strand DNA replication is continuous but on the lagging strand it is discontinuous and performed in Okazaki fragments. (C) The ultimate ORI on the lagging strand. (D) The ultimate primer cannot be replaced and, therefore, the lagging strand shortens with every cell division by a few base pairs if no repair mechanisms are activated.

The presence of telomeres at the ends of chromosomes prevents the loss of coding genes. Within limits, the loss of telomeric DNA has minor consequences for the cell. However, more substantial telomere loss is associated with cellular ageing *in vitro*. In 1961 Leonard Hayflick observed that cells in culture are not able to divide endlessly (Hayflick & Moorhead 1961). After approximately 60 cell divisions (the so called “Hayflick limit”) they enter replicative senescence which is a state of the cell where it is still viable but unable to divide further (Hayflick & Moorhead 1961; Goldstein 1990). Later it was shown that telomeres shortened with the number of cell divisions *in vitro* and that telomere shortening correlated with replicative senescence (Harley et al., 1990). A causal relationship between telomere shortening and replicative senescence *in vitro* was established when the expression of the

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enzyme telomerase (see below) in retinal pigment epithelial cells and foreskin fibroblasts was shown to elongate telomeres and, at the same time, extend the proliferative lifespan of such cells (Bodnar et al. 1998). This result has since been supported by other studies (Jiang 1999; Simonsen et al. 2002). When telomeres are critically short and no telomere elongation mechanisms are activated, a DNA damage response is triggered that leads to apoptosis or senescence (Fagagna et al. 2003). Which pathway is activated seems to depend on the affected cell type and the activity of tumour suppressor genes such as P53 (De Lange 2005; Stewart et al. 2012). Fibroblasts often enter replicative senescence while lymphocytes usually undergo apoptosis (Karlseder 1999; De Lange 2005). Apoptosis describes the programmed cell death which does not cause an inflammatory reaction (Kerr et al., 1972). A senescent cell, on the other hand, survives but its cell cycle is permanently arrested (Goldstein 1990; Hayflick & Moorhead 1961). Replicative senescence might be a tumour prevention mechanism, because cells with a long replicative history and therefore a greater potential for mutations are removed from the mitotic pool similar to cells that undergo apoptosis. On the other hand senescent cells might become malfunctioning and trigger inflammation processes or promote tumour development (Blackburn & Epel, 2012). It has been established that telomere shortening leads to replicative senescence *in vitro*. However, even before inducing replicative senescence, the cellular signalling of telomeres might change depending on their length. As described above the telomere position effect seems to be TL dependent and leads to an altered expression of genes (Baur et al. 2001; Robin et al. 2014). It was suggested that telomerase itself might be activated by telomere position effect over long distance (Kim et al., 2016).

1.1.4. Telomerase

Telomerase is a ribonucleoprotein complex with reverse transcriptase activity (Greider & Blackburn, 1985). It consists of two major sub-units: The catalytic centre (TERT) (Lingner 1997; Harrington et al. 1997; Nakamura et al. 1997) and an RNA template (TERC) (Feng et al., 1995; Greider & Blackburn, 1989). Additionally, many other proteins with largely unknown functions are bound to the telomerase complex (reviewed by Cong et al., 2002). Telomerase can elongate telomeres by using its own RNA template to add telomeric sequences to the 3' end of the telomere. When the 3' end is sufficiently elongated, a primer is synthesised and the complementary strand is replicated by a DNA polymerase. While telomerase is active in embryonic

cells, adult germ cells and many cancer cells, it is usually repressed in differentiated tissues (Kim et al., 1994).

1.1.5. Telomere length regulation

TL seems to be tightly regulated at a cellular level. Short telomeres can be replenished by the enzyme telomerase (Greider & Blackburn, 1985) and an additional telomerase-independent mechanism of telomere elongation termed Alternative Lengthening of Telomeres (ALT) was first described in cancer cells (Bryan et al., 1997; Cesare & Reddel, 2008, 2010; Henson et al., 2002; Londoño-Vallejo et al., 2004). However, there is some evidence that ALT might also be activated in normal mammalian cells for telomere maintenance (Neumann et al. 2013). ALT involves homologous recombination and exchange of telomeric DNA between chromosome ends (Cesare & Reddel 2010; Henson et al. 2002; Cesare & Reddel 2008).

Additionally to factors that passively shorten telomeres such as oxidative stress and UV A irradiation (von Zglinicki 2002; Oikawa et al. 2001), a pathway that seems to be actively controlled by the cell and leads to fast telomere shortening, also known as telomere trimming, has been described (Li et al. 2017; Pickett et al. 2009; Pickett et al. 2011; Pickett & Reddel 2012). A relatively low concentration of shelterin proteins at long telomeres is believed to activate the protein TZAP which leads to a fast deletion of telomeric sequence (Li et al. 2017). The activity of TZAP is increased in ALT positive cells that show fast telomere elongation, which indicates the presence of a negative feedback loop between the length of telomeres and the activation of telomere trimming. Short telomeres, on the other hand, are suspected to trigger telomere elongation through the telomere position effect over long distance and the activation of TERT (Kim et al., 2016). This again implies a negative feedback loop between TL, telomerase expression and thus telomere elongation.

1.1.6. Tissue selection for telomere length measurement

After TL was shown to be associated with cellular ageing in vitro (Bodnar et al., 1998; Harley et al., 1990), naturally the interest in a potential association of TL with organismal ageing and lifespan was sparked. Many early studies were cross-sectional where TL in samples of young individuals were compared to samples of different, old individuals, without considering repeated samples of the same individual at different ages. The advantage of this approach was that it allowed the use of tissues that are not easily accessible without invasive procedures such as

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brain tissue (Allsopp et al. 1995); the disadvantage was that the method did not allow the distinction between among and within individual variation of TL and TL dynamics with age.

Over the years blood cells have been proposed as an accessible tissue for the measurement and study of TL, allowing repeated samples per individual. The latter is a requirement in studies aiming to better understand telomere dynamics within individuals over lifetime. Even though blood cell TL may differ from TL in other tissues, the correlation of TL from different tissues is quite high as shown in studies on humans, cattle and Zebra finches (Friedrich et al., 2000; Laubenthal et al., 2016; Reichert et al., 2013). Therefore, blood cell TL may act as a surrogate for TL in other tissues. In birds and reptiles, erythrocytes are nucleated and, because of their predominance in a blood sample, telomeres are mainly measured within red blood cells in these species. In contrast erythrocytes in mammals contain no nucleus, no DNA and therefore no telomeres. In mammal species, TL is therefore measured in leukocytes if blood cells are used. Leukocytes have the same advantages as red blood cells in that they are easily accessible and possible to sample repeatedly. However, the disadvantage of leukocytes is that they consist of different cell types (macrophages, granulocytes, lymphocytes) that are known to differ in TL (Baerlocher et al., 2007; Kimura et al., 2010; Lin et al., 2010b, 2016). The composition of circulating leukocytes may change in response to stress or inflammation and TL might be altered as a consequence. Therefore changes in leukocyte TL are more complex to explain. Despite this complication leukocytes are the most frequently studied cell type in longitudinal telomere studies in mammals so far.

1.1.7. Telomere length in humans

Soon after the discovery that TL changes with the number of cell divisions *in vitro*, it was shown that leukocyte TL in humans declines with age *in vivo* as well (Hastie et al. 1990). However, TL is highly variable among individuals of the same age group. In fact, the distribution of TL in new-borns often overlaps the distribution in adult individuals (Aubert & Lansdorp 2008). TL *in vivo* is influenced by genetic and environmental factors. In humans, TL is determined by many different genes that all contribute a small effect to the phenotype (Pooley et al. 2013; Gatbonton et al. 2006; Levy et al. 2010; Mangino et al. 2008; Vasa-Nicotera et al. 2005). The heritability of TL was estimated to be between 0.39 and 0.82 (Njajou et al. 2007; Hjelmborg et al.

2015; Broer et al. 2013; Bischoff et al. 2005). Environmental effects that have been found to influence TL include oxidative stress (von Zglinicki 2002), psychological stress (Epel et al., 2004; Shalev et al., 2012), socio-economic status (Cherkas et al., 2006), body mass index (Kim et al., 2009; Mirabello et al., 2009), smoking status (Huzen et al. 2014; Mirabello et al. 2009; Ehrlenbach et al. 2009), diet (Cassidy et al., 2010; Nettleton et al., 2008), relaxation techniques (Epel et al., 2009; Hoge et al., 2013) and level of physical exercise (Cherkas et al., 2008).

Telomere change across age has been described to follow a general pattern with three stages: 1) fast telomere attrition in early life, 2) slower attrition or plateau in adult life, 3) second stage of faster attrition at old ages (Aubert & Lansdorp 2008; Frenck et al. 1998). While some studies suggest that over the course of life the rank order of individuals remains similar so that individuals with long telomeres at the beginning of life also have relatively long telomeres at old age (Benetos et al. 2013), other studies suggest that environmental effects can impact TL to an extent that the rank order of individuals may change (Blackburn et al., 2015).

Both long and short telomeres have been shown to be associated with diseases in humans. On one hand there are genetic disorders that lead to short telomeres and specific telomere syndromes, on the other hand physiological variation of TL within age groups has been associated with different diseases.

Telomere syndromes are the consequence of mutations within genes that encode the telomerase complex. Even though telomerase expression is difficult to detect in most differentiated cells, a low level of activity seems to be important to maintain normal cellular physiology (Greider, 2016). In humans, several mutations within telomerase genes have been shown to cause genetic diseases that include dyskeratosis congenita, bone marrow failure and idiopathic pulmonary fibrosis (reviewed by Garcia et al., 2007). Dyskeratosis congenita is a severe multisystem disorder (Garcia et al. 2007; Dokal 2000). Its symptoms are mainly associated with tissues that demonstrate high stem cell turnover rates and include but are not restricted to skin, nail and pulmonary abnormalities. The leading causes of death are bone marrow failure, pulmonary complications and cancers (Dokal 2000). Death occurs usually before the age of 20 years (Dokal 2000). Some mutations within the telomerase genes have been linked to bone marrow failure in patients that do not show other clinical symptoms of dyskeratosis congenita (Garcia et al. 2007). Also, mutations within telomerase genes have been found in families with a high

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prevalence of idiopathic pulmonary fibrosis, a fatal disease that is associated with lung scarring (Garcia et al. 2007).

Telomerase activity and a controlled TL seem to be required for the organism to remain healthy. Telomerase deficiency is associated with symptoms that usually affect older generations. This observation is supported by results from studies in TERT-knockout mice that were followed over six generations and showed decreased proliferative capacity of hematopoietic stem cells and stem cells in spleen and testes, chromosomal abnormalities, slower wound healing, higher tumour incidences and a reduced lifespan (Blasco, 1997; Greider et al., 1998; Rudolph et al., 1999).

Next to telomere syndromes, extremely long and extremely short telomeres within the normal physiological range have been associated with diseases and mortality in humans (Stanley & Armanios 2015). Short telomeres were associated with an early onset of age related symptoms and diseases such as type 2 diabetes mellitus (Salpea et al., 2010; Zhao et al., 2013), cardiovascular disease (Yeh & Wang 2016; Haycock et al. 2014; Aviv 2012), cognitive impairment (Hägg et al., 2017; Hochstrasser et al., 2011), brain atrophy (Wikgren et al. 2014) and overall cancer incidence (Willeit et. al, 2011). Additionally, short telomeres were linked to a shorter lifespan (Bakaysa et al., 2007; Cawthon et al., 2003). In contrast, long telomeres were associated with a higher risk of specific cancer types such as melanomas (Stanley & Armanios 2015). Next to studies showing a meaningful correlation between TL, disease and mortality there are also studies that find no such relationships. For example one study did not find a correlation between TL and type 2 diabetes mellitus (Hovatta et al. 2012) and other studies did not reveal a relationship between TL and cardiovascular disease (Raymond et al., 2012) or mortality (Harris et al., 2006; Martin-Ruiz et al., 2005). Despite the mixed results reported so far, TL has been discussed as a biomarker for health, ageing and longevity in humans (Bekaert et al., 2005; Cawthon et al., 2003; Mather et al., 2011; Sanders & Newman, 2013; Simm et al., 2008). More longitudinal studies are required to better understand the dynamics of TL and their association with diseases and mortality, and to investigate the potential use of TL as a biomarker for health and longevity.

1.1.8. *Telomere length in species other than humans*

Outside of human biomedical and epidemiological research, telomere studies are popular in the field of evolutionary ecology. Studies in different species and taxa underline how much TL and TL dynamics vary among different living organisms. In simple organisms such as yeast and ciliates only a few hundred base pairs of telomeric DNA are sufficient for telomere function (Blackburn, 1991). In vertebrates, TL ranges from approximately five thousand base pairs in sea lions to more than 50,000 base pairs in tigers, rabbits and mice (Gomes et al. 2011).

Short telomeres were associated with a longer lifespan ($p=0.003$) across more than 60 different mammalian species (Gomes et al. 2011). However, opposite results regarding the direction of the relationship between TL and lifespan have been reported within species such as Soay sheep, tree swallows and zebra finches (Fairlie et al., 2015; Hausmann et al., 2005; Heidinger et al., 2012). Faster telomere attrition rate was found to be associated with shorter maximum lifespan across several avian and mammal species (Hausmann et al., 2003).

Telomere dynamics and the association between TL and survival have been intensely studied in birds (Hausmann et al., 2005; Heidinger et al., 2012; Nettle et al., 2013; Voillemot et al., 2012). In many avian species such as Adélie penguins, common terns, tree swallows, zebra finches and great frigatebirds TL declines with age (Hausmann et al., 2003; Juola et al., 2006). However, in European shags and wandering albatrosses, telomeres shorten only at a young age and remain relatively constant in adult life (Hall et al. 2004). In long-lived Leach's storm petrels, TL was even found to be shorter in nestlings than in older birds (Hausmann et al., 2003). This might be due to higher telomerase expression in Leach's storm petrels' bone marrow compared to other birds (Hausmann et al., 2007) or due to selective disappearance of animals with short telomeres (Hausmann & Mauck, 2008). Studies investigating the relationship between avian TL and longevity reported the following: Wild tree swallows with long telomeres at the age of one year lived longer than their peers with short telomeres (Hausmann et al., 2005). Also, in one of the few lifelong longitudinal telomere studies published so far, captive Zebra finches with long telomeres at birth maintained long telomeres over their lifetime and survived for longer (Heidinger et al. 2012). Furthermore, faster telomere attrition rate was correlated with shorter lifespan in Alpine swifts, jackdaws and Seychelles warblers (Barrett et al., 2013; Bize et al., 2009; Boonekamp et al., 2014).

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Similar to humans environmental factors were reported to affect TL in birds as well: American redstarts that wintered in a low-quality habitats showed more telomere attrition than birds that wintered in high-quality habitats (Angelier et al. 2013). In black-legged kittiwakes, induced stress during reproduction led to faster telomere attrition while more time spent at the wintering grounds was associated with less telomere attrition (Schultner et al. 2014). In great reed warblers, chronic malaria infection was correlated with faster telomere attrition, poorer reproduction and a shorter lifespan (Asghar et al. 2015). Most of those results seem to have in common that they are likely to be mediated by stress responses. Experimental induction of stress in several bird studies has demonstrated that cellular stress may be linked to telomere shortening: In domestic chicken, embryonic exposure to a high dose of the stress hormone cortisol was associated with shorter telomeres in 21 day old chicks (Hausmann et al. 2012). In European starlings the experimental change of brood sizes showed that birds with more dominant food competitors in the same nest had a faster telomere attrition rate (Nettle et al. 2013). This effect persisted into adulthood. Other studies supported the association of stress exposure in early life and shorter telomeres (Herborn et al. 2014; Nettle et al. 2017)

While the environmental effects on TL are intensely studied in birds, there is not much known about the genetics of avian TL. Two studies reported heritability estimates which for kakapos and collared flycatchers which were 0.42-0.77 and 0.09 respectively (Horn et al. 2011; Voillemot et al. 2012).

In fish, telomere dynamics are very different across and within species: One study in zebra fish reported that telomeres lengthened in the beginning of life and shortened in later adulthood (Anchelin et al., 2011). However, a different study in zebra fish found no relationship between age and TL (Lund et al., 2009). Similar results were reported for Atlantic silversides, whereas in Japanese medakas TL shortens with age similarly to humans (Hatakeyama et al. 2008). Different TL change patterns within and across fish species might be explained by different telomerase expression levels throughout life (Anchelin et al. 2011; Hatakeyama et al. 2008).

A few studies have been published about TL dynamics over life in reptiles: Tropical pythons first elongate their telomeres in early life and shorten them after an approximate age of six years (Ujvari, 2009). There was no relationship between blood cell TL and age in loggerhead sea turtles (Hatase et al. 2008). In *Alligator*

mississippiensis, TL shortened with body length as a proxy for age (Scott et al. 2006). In female sand lizards age and TL were positively correlated while there was no relationship in male animals (Olsson et al. 2010). The heritability of TL in sand lizards was 0.52 (Olsson et al. 2011).

Studies in non-human and non-model mammals, particularly longitudinal studies are relatively scarce. Some mammal species follow a similar telomere shortening pattern over life as humans. In dogs, cats, sheep, goats and badgers for example, telomeres shorten with age (Brümmendorf et al. 2002; Benetos et al. 2011; Alexander et al. 2007; Betts et al. 2005). However, in baboons TL remains stable after attrition in early life (Baerlocher et al., 2007). Edible dormice show interesting deviations from telomere dynamics observed in other species: After the initial decline in early age, telomeres elongate considerably (Hoelzl et al. 2016). One life-long telomere study in feral Soay sheep reported complex dynamics with age, where telomeres shortened in the beginning of life, elongated thereafter and shortened later in life again (Fairlie et al. 2015). While telomere shortening was due to telomere changes within the individual, telomere elongation might have been due to selective disappearance of animals with short telomeres (Fairlie et al. 2015). Soay sheep lambs with long telomeres were more likely to survive their first winter (Fairlie et al. 2015).

1.1.9. Telomere length in livestock species

The knowledge about TL and TL dynamics in livestock species is limited so far. Most studies were performed on cloned sheep, pigs, goats and cattle (Miyashita et al. 2002; Betts et al. 2005; Alexander et al. 2007; Jeon et al. 2005). A few studies specifically in cattle have shown that TL varies across breeds (Tilesi et al. 2010) and tissues (Laubenthal et al. 2016). In two cross-sectional studies, bovine TL was shorter in older animals (Brown et al., 2012; Miyashita et al., 2002). In a study on 21 German Holstein cows aged 2 to 6 years, blood samples were taken in early and late lactation (single lactation per cow) and it was shown that TL shortened over the course of lactation (Laubenthal et al. 2016). However, the authors may not have sufficiently controlled for the age effect on TL. In the same study bovine leukocyte TL correlated well with TL in subcutaneous fat, liver and mammary gland cells (Laubenthal et al. 2016). This suggests that leukocyte TL in cattle may be used as a proxy for TL in other bovine tissues. A single study so far reported that cows with short telomeres were more likely to be culled within the next year which implies that

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there might be a relationship between TL and survival in a producing herd of dairy cows (Brown et al. 2012). There is growing interest in testing TL as a biomarker for productive lifespan and animal welfare in dairy cattle (Brown et al. 2012; Bateson 2016).

From all those studies across different species and different vertebrate classes, it is obvious that telomere dynamics are complex. While telomere shortening with age seems to be the rule, there are significant deviations and a general telomere shortening paradigm is not valid for all species and individuals. Also, the relationship between TL and survival is not totally clear. However, a recent meta-analysis including mostly birds, but also reptiles and Soay sheep reported that, overall, there was a negative relationship between TL and subsequent mortality risk across bird studies (Wilbourn et al. 2018).

In all cases, there is a high demand for more longitudinal studies of telomere dynamics, particularly for lifelong studies that include repeated samples on the same individuals across time. Also, more genetic studies outside the human literature are needed to better understand the biology of TL and its possible application as a biomarker in animal genetic improvement programmes. There are no heritability estimates of TL in a livestock species available so far.

1.1.10. Methods for telomere length measurement

Different laboratory methods have been developed for the measurement of TL and a suitable measurement method has to be chosen based on the aims and requirements of a particular study (Nussey et al. 2014; Aubert et al. 2012).

The terminal restriction fragment (TRF) assay was developed almost thirty years ago (Harley et al. 1990) and has been considered for many years the “gold standard” for measuring TL. First, it was the only available measurement technique, and then it was used to validate all new TL measurement methods (Aubert, et al. 2012). Steps of TL measurement by TRF include the digestion of non-telomeric DNA with restriction enzymes, sorting of telomeric sequences by size using gel electrophoresis and detection of telomeric sequences either with southern blotting (Kimura et al. 2010) or in-gel labelling (Hausmann & Mauck 2008) with a telomere specific probe. Specific software is required to translate the visual data of the resulting telomeric smear to quantitative measurements such as mean, median and range of TL within a sample (Nussey et al. 2014; Aubert et al. 2012). The

measurement of not only the mean TL but also the variance with TRF is one of the strengths of this method (Kimura et al. 2010; Haussmann & Mauck 2008; Nussey et al. 2014). Also, TRF measures TL in the absolute unit of kilobases which makes a comparison of TL across different studies and species possible. However, the comparability of results is limited by the fact that TRF protocols are not standardized across laboratories and that factors such as the choice of restriction enzymes or the image analysis software can influence the results (Aubert et al. 2012). A disadvantage of TRF is that it is relatively laborious and expensive when compared to a qPCR based assay. Also, it measures not only pure telomeric repeats but also so-called subtelomeric regions and telomere variant repeats which may lead to an overestimation of TL. Even though TRF informs about the variance of TL in a sample it is not particularly sensitive for the detection of the shortest telomeres (Aubert et al. 2012). It is likely that not average TL but the accumulation of critically short telomeres in a cell triggers replicative senescence (Hemann et al. 2001) and therefore might be of particular interest for many studies. The single telomere length analysis (STELA) combines qPCR amplification of telomeric DNA at specific chromosome ends and southern blotting techniques and is better suited for the detection of short telomeres (Baird et al. 2003). But STELA is even more laborious and time consuming than TRF and, therefore, predominantly used in projects that require a superb resolution such as studies in human cancer research (Jones et al. 2012; Xu & Blackburn 2007; Roger et al. 2013; Lin et al. 2010). Different fluorescence in situ hybridisation (FISH) techniques (Lansdorp et al. 1996; Rufer et al. 1998) have the advantages that they can detect TL at specific chromosome ends, are able to measure critically short telomeres and allow sorting leukocytes into their sub-populations which enables a separate measurement of TL in different white blood cell types and the detection of a change in leukocyte composition. However, those techniques require viable cells, fixed tissues or fresh blood samples (Nussey et al. 2014).

A popular technique for the measurement of relative telomere length (RTL) is a qPCR based assay, where TL is measured as the amount of telomeric DNA in relation to the amplification of a reference gene that is non-variant in copy number (Cawthon, 2002). Therefore, only an average TL per genome within the sample can be estimated while the variance of TL remains unknown. For comparability of samples located on different qPCR plates, an identical sample (the “calibrator” or “golden sample”) is repeated on all qPCR plates in the study and measurements for

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the calibrator are included in the calculation of RTL of the individual sample. The relative units of RTL make a comparison across species and studies extremely challenging. Even if exactly the same qPCR protocol with the same primers for the telomere and reference gene amplification was used, a comparison of studies would probably only be possible within the same species or across species that share the same number of chromosomes and the same amount of reference gene sequence. Another disadvantage of qPCR is that measurements are less precise than with other methods. The noise can be reduced by measuring each sample in duplicates or triplicates and by ensuring a high standard of DNA purity and integrity. Clear advantages of qPCR compared to TRF are that it requires less DNA and is faster and more economical. It is a widely accepted measurement method predominantly used in large scale telomere studies.

A literature survey of telomere studies in vertebrates other than laboratory model species and humans revealed that 60 of 63 such studies between the years 2002 and 2015 used either TRF (TRF; Hausmann & Mauck, 2008; Kimura et al., 2010) or qPCR (Cawthon, 2002) methods to measure TL (Appendix A, Table 1). The number of telomere studies has increased substantially over that time and the qPCR method has gradually gained popularity (Figure 3 A). This is probably due to a shift to large-scale longitudinal studies and due to qPCR being faster, cheaper and requiring less DNA than TRF (Nussey et al. 2014). In parallel with an increase in the use of the qPCR method, silica based DNA extraction methods gained popularity (Figure 3 B and Appendix A, Table 1). A total of 75% of the qPCR-based TL studies used silica membrane-based DNA extraction methods, presumably because both represent high throughput techniques.

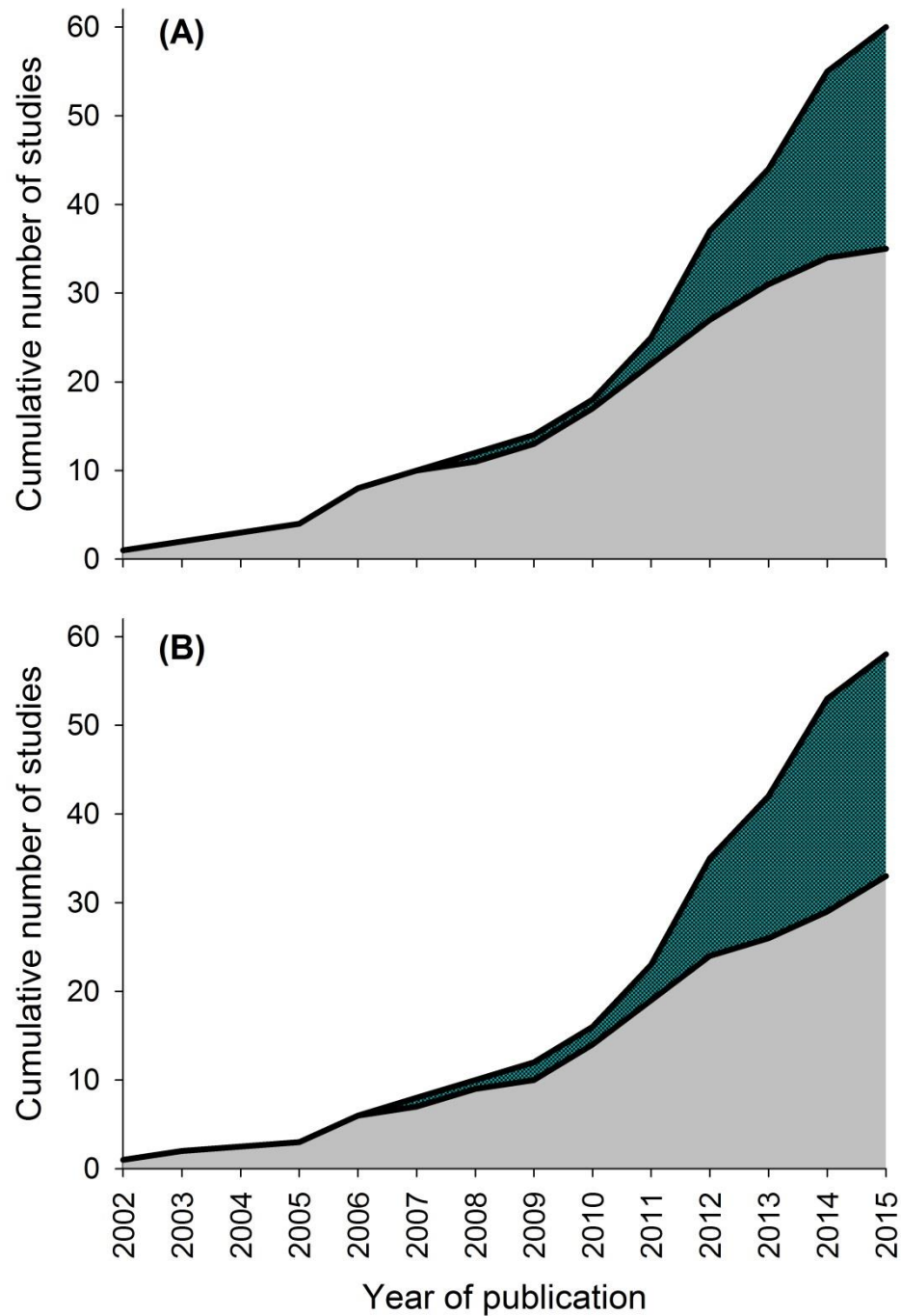


Figure 3: Number of published studies between 2002 and 2015 on (A) telomere length measured by TRF (grey area) and qPCR (dotted green area) and (B) DNA extraction based on non-silica membrane (grey area) or silica membrane (dotted green area) methods in vertebrates other than humans and laboratory animals.

1.2. Longevity in producing dairy cattle

Dairy cattle are commercially used animals reared for milk production. Cows are typically kept on the farm until they are removed (culled) by the farmer. Therefore,

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longevity of dairy cattle, as for any other livestock species, has to be defined differently than for free-living populations. The decision to cull a cow can be dictated by any of many possible reasons. Depending on the reason, the removal of a cow from the herd can be described as voluntary or involuntary culling. Voluntary culling describes cases where the farmer decides to replace an old low producing cow by a higher producing, usually younger cow. Ideally cows remain in the herd for at least three lactations in order for the farmer to retrieve rearing costs and generate some profit (Figure 4). However, in many cases, farmers are forced to remove cows from their herds earlier due to fertility problems or diseases; this is known as involuntary culling. The fact that a large number of heifers (cows before first calving) never reach production age and dairy cows in production are culled involuntarily early in their life causes severe losses to the dairy industry. The costs for replacing culled cows are typically the second highest variable costs after feed and forage for a dairy farm.

Improving survival of both replacement heifers and dairy cows in production would save money and resources, benefit the environment and improve animal welfare. A better productive lifespan of dairy cows would reduce the requirement for replacement heifers which is associated with a reduction in greenhouse gas emission and requirements for feed and grazing land. Animal welfare would improve because breeding would focus on dairy cattle that can cope better with the metabolic demands of high milk yield while staying healthy for a long time. Therefore, improving the productive lifespan of dairy cattle will enhance sustainable production, reduce wastage and support the milk supply for the growing world population on limited agricultural space.

Genetic improvement of productive lifespan is difficult, because the trait is measured late in life and is only available for females (Visscher et al., 1999). Furthermore, longevity traits are known to have a low heritability. In an analysis of national UK data, the heritability estimates for heifer survival was ~ 0.01 and for cow survival ~ 0.06 (Pritchard et al., 2013). Phenotypes on reasons for culling are often imprecisely recorded which makes it difficult to interpret how many cows are culled voluntarily and how many involuntarily.

Early life biomarkers associated with longevity would facilitate programmes aiming at enhancing cow productive life. Given their established association with cellular

ageing (Harley et al. 1990; Bodnar et al. 1998; Jiang 1999; Simonsen et al. 2002), it would be useful to examine TL as an early life biomarker to predict longevity and apply this knowledge to breeding schemes.

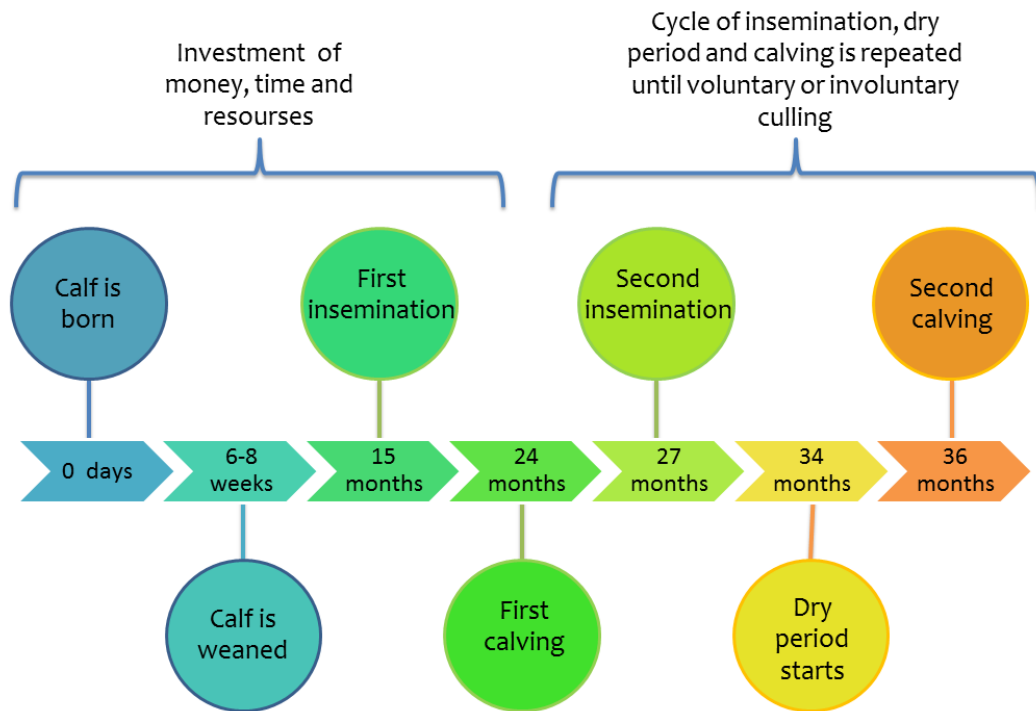


Figure 4: Productive lifespan of a dairy cow.

1.3. Animal Population for this thesis

The Langhill herd that is kept at the Crichton Royal research farm in Dumfries (Scotland, UK) was used as the resource population in this thesis. The herd has been intensely studied over the last 30 years. Two distinct genetic lines are managed on two different diets to allow genetic and nutritional scientific studies. One genetic line is bred to perform at the UK top level regarding milk fat and protein yield (select line), whilst the other genetic line is deliberately maintained at UK average performance level (control line). At the time of first calving cows of both genetic lines are randomly allocated to two different diets: One is rich in calories and low in forage and the other contains less energy and a higher proportion of forage

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(Veerkamp et al. 1994). For further details about the Crichton herd, please refer to Appendix B of this thesis.

For this thesis we selected 1,328 samples of 308 animals that were equally distributed over all genetic and feeding groups. Repeated blood samples were available on all animals that were used to measure TL at different ages. All animals were first blood sampled within 15 days after birth. Animals that were alive at time of sample selection for this thesis had to have at least four blood samples to be included in the study. To reduce possible bias, animals that were dead at the time of sample selection only had to have two samples to be included in the study. This allowed the inclusion of all animals that died early in life, before providing a third and fourth sample.

Additionally, 284 samples of 38 female calves were also used. These samples were taken at an approximately monthly interval to enable the investigation of early life TL dynamics in more detail.

1.4. Thesis objectives

The objectives of this thesis were

- 1) the development of robust methods for the measurement of bovine leukocyte TL in cattle,
- 2) the examination of change in bovine leukocyte TL across time at a population level and at an individual animal level,
- 3) the estimation of genetic parameters of bovine leukocyte TL and
- 4) the assessment of the association between bovine leukocyte TL profiles across time with productive lifespan of dairy cattle.

1.5. Thesis outline

In Chapter 2 a robust and reliable method for the measurement of bovine leukocyte TL length with qPCR is presented. The effect of different DNA extraction protocols on bovine leukocyte TL measurements is tested and a method of choice is identified which is used throughout this study.

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Chapter 3 describes bovine leukocyte TL dynamics at a population level. Mixed models are used to test which factors influence bovine leukocyte TL measurements and calculate overall heritability estimates. The association between bovine leukocyte TL measured at different ages with productive lifespan is investigated.

Chapter 4 illustrates change of bovine leukocyte TL within individual animals across time defined by age using random regression models. Genetic variance and heritability are estimated at different ages and their change over time is explored. The genetic correlation between bovine leukocyte TL measurements across time is calculated. Differences in telomere change patterns are investigated in relation to productive lifespan.

Chapter 5 takes a closer look at intra-individual change following up on Chapter 4. For each animal the mean bovine leukocyte TL over the sampling period as well as mean TL change and mean absolute TL change are analysed in relationship with productive lifespan.

Chapter 2

Method specific calibration corrects for DNA extraction method effects on relative telomere length measurements by quantitative PCR

2.6. Prefix

In this chapter robust methods for the measurement of RLTL in cattle that are applicable to a large-scale project are developed. The effect of silica based DNA extraction methods on RLTL is thoroughly tested.

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My contributions to this paper were as follows: I participated in the planning of the experiment, the extraction of DNA, measurement of RLTL and I was responsible for the data analysis. I wrote the first draft of the manuscript and revised it with the help of my supervisors and co-authors.

Supplementary files S1 and S2 of the publication are inserted in the end of this chapter as chapter appendix 1 (0) and chapter appendix 2 (2.16).

2.7. Abstract

Telomere length (TL) is increasingly being used as a biomarker in epidemiological, biomedical and ecological studies. A wide range of DNA extraction techniques have been used in telomere experiments and recent quantitative PCR (qPCR) based studies suggest that the choice of DNA extraction method may influence average relative TL (RTL) measurements. Such extraction method effects may limit the use of historically collected DNA samples extracted with different methods. However, if extraction method effects are systematic an extraction method specific (MS) calibrator might be able to correct for them, because systematic effects would influence the calibrator sample in the same way as all other samples. In the present study we tested whether leukocyte RTL in blood samples from Holstein Friesian cattle and Soay sheep measured by qPCR was influenced by DNA extraction method and whether MS calibration could account for any observed differences. We compared two silica membrane-based DNA extraction kits and a salting out method. All extraction methods were optimized to yield enough high quality DNA for TL measurement. In both species we found that silica membrane-based DNA extraction methods produced shorter RTL measurements than the non-membrane-based method when calibrated against an identical calibrator. However, these differences were not statistically detectable when a MS calibrator was used to calculate RTL. This approach produced RTL measurements that were highly correlated across extraction methods ($r > 0.76$) and had coefficients of variation lower than 10% across plates of identical samples extracted by different methods. Our results are consistent with previous findings that popular membrane-based DNA extraction methods may lead to shorter RTL measurements than non-membrane-based methods. However, we also demonstrate that these differences can be accounted for by using an extraction method-specific calibrator, offering researchers a simple means of accounting for differences in RTL measurements from samples extracted by different DNA extraction methods within a study.

2.8. Introduction

Telomere shortening has recently been identified as one of nine 'hallmarks of aging' (López-Otín et al. 2013) and blood cell telomere length (TL) is an increasingly widely measured biomarker in human epidemiology and vertebrate ecology (E. H. Blackburn et al. 2015; Monaghan & Haussmann 2006; Monaghan 2014). Many methods are available to measure TL, each with their own strengths and drawbacks

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(Aubert & Lansdorp 2008; Nussey et al. 2014). Quantitative PCR (qPCR)-based methods have become increasingly popular in recent years, presumably due to their being faster, cheaper and requiring less DNA than most other methods (Aubert & Lansdorp 2008; Nussey et al. 2014). However, the qPCR method has drawbacks, notably a lower repeatability compared to terminal restriction fragment (TRF) southern blot (Aviv et al. 2011; Martin-Ruiz et al. 2014) and the relative units of measurement, which makes comparison across studies and species extremely challenging (Aubert & Lansdorp 2008; Aviv et al. 2011) if not impossible. Furthermore, there is mounting recent evidence that relative TL (RTL) measurements by qPCR may be influenced by methods of sample acquisition and storage (Zanet et al. 2013) and DNA extraction methods (Cunningham et al. 2013; J. N. Hofmann et al. 2014; Denham et al. 2014; Tolios et al. 2015; Raschenberger et al. 2016). Understanding how such methodological variation may influence RTL measurements by qPCR both within and among laboratories is essential for evaluating and comparing results of telomere studies.

A central requirement of all methods of TL measurement is the extraction of a suitable quantity of high quality DNA. A considerable number of DNA extraction methods have been employed to date by researchers studying TL (Cunningham et al. 2013). In general two different types of DNA extraction methods can be distinguished: One uses a solid phase such as silica membranes or magnetic beads. DNA binds to the solid phase, is washed and then eluted. The other type is based on the transition of DNA between different solvents. Those methods (for example salting out or phenol-chloroform extractions) do not require a solid phase. The question that arises from the literature is whether solid phases act as physical barriers that shear DNA and therefore cause shorter TL measurements. Two recent studies using human blood samples with the qPCR method suggested that silica membrane-based DNA extraction methods yield shorter RTL measurements than other methods (Cunningham et al. 2013; Hofmann et al. 2014). Two further studies have reported differences in mean TL from DNA extracted using a range of different methods, although these differences were not specifically linked to the use of silica membranes (Denham et al. 2014; Tolios et al. 2015). Recently, another study found that RTL from samples extracted by a magnetic bead method was shorter when compared to salting out and phenol chloroform (Raschenberger et al. 2016). Although it is obviously desirable to keep methodology as consistent as possible, potentially valuable and informative archived DNA samples may be available to

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researchers interested in telomere dynamics which may not have been extracted by the same technique. In such cases, understanding and potentially accounting for the effects of extraction method on TL measurement is essential (Tackney et al. 2014). Furthermore, a better understanding of such methodological effects could help ensure appropriate aspects of DNA preparation methodology are accounted for in meta-analyses of TL studies (Cunningham et al. 2013).

The qPCR method measures RTL as the total amount of telomeric sequence relative to the amount of a non-variable copy reference gene sequence within the same DNA sample (Cawthon 2002). Standard methods for calculating RTL require a calibrator sample (also called “reference sample” (Cawthon 2002) or “golden sample” (Nussey et al. 2014)), which is an identical DNA sample included on every qPCR plate for both telomere and reference gene reactions. Sample RTL is expressed relative to the calibrator to account for random measurement error and resulting plate-to-plate variation. A wide range of samples have been used as calibrators: DNA from a chosen individual, pooled DNA from several individuals (Cawthon 2002) or commercially available DNA (Raschenberger et al. 2016). Previous studies examining effects of DNA extraction method on RTL appear to have used a single calibrator, extracted by one identical method (Cunningham et al. 2013; Tolios et al. 2015; Hofmann et al. 2014; Denham et al. 2014; Raschenberger et al. 2016). They observed extraction method dependent differences in RTLs that in some studies appear to be not random but systematic (Cunningham et al. 2013; Hofmann et al. 2014; Raschenberger et al. 2016). In principle, it should be possible to account for such systematic extraction method effects by taking the same calibrator sample and extracting DNA from it using different methods to match the methods used on the samples in the study. With this approach, the calibrator should be influenced in the same direction and to a similar degree by the extraction method. Using such a DNA extraction method specific calibrator in RTL calculations, could therefore adjust for any effect of extraction method on the samples’ telomere length. The effectiveness of this approach has yet to be tested.

The objective of the present study was to assess the effect of two different DNA extraction methods, and the use of different calibrators on RTL measurements. We compared RTL measurements of blood samples that were collected from a Holstein Friesian cattle population after extracting DNA using two silica membrane-based DNA extraction protocols and a salting out (non-membrane-based) method. To

validate our results with samples from a different species we compared one of the two silica membrane-based methods with the salting out method using buffy coat samples from wild Soay sheep. We found high repeatability of RTL measurements, regardless of DNA extraction method, and no difference in mean RTL among extraction methods when a DNA extraction method specific (MS) calibrator was used.

2.9. Materials and Methods

2.9.1. Study systems & sampling

Whole blood samples were collected from Holstein Friesian cattle during 2009-2013 at the Crichton Royal Farm (Dumfries, Scotland) as part of a long-term genetics study for which blood samples have been archived for many years (Veerkamp et al. 1994). Samples were taken by venepuncture using EDTA as anticoagulant and were stored at -30°C until DNA extraction. We selected 72 samples from animals among which both sexes and a range of ages were represented (45 females aged 0-9 years and 27 male new-born calves).

Additionally, we used blood samples collected from a wild population of Soay sheep on the St Kilda archipelago in the Outer Hebrides (Scotland), which have been subject to individual-based monitoring and regular sampling since 1985 (Clutton-Brock & Pemberton 2004). Blood samples were taken by venepuncture in August 2013, using heparin as an anticoagulant. Buffy coat fractions were prepared as follows: whole blood samples were centrifuged at 3,000 rpm for 10 minutes. The plasma layer was removed and remaining cells were washed by adding 0.9% NaCl solution. After centrifugation for 10 minutes at 3,000 rpm the intermediate buffy coat layer was collected, transferred to a 1.5 ml Eppendorf tube and stored at -20°C until further use. We selected samples from 48 different females aged 4-13 years for DNA extraction.

2.9.2. Ethics statement

Blood sampling from Holstein Friesian cattle and Soay sheep was approved by the Animal Experiments Committee (UK Home Office Project License Numbers: PPL 60/4278 and 60/3547, respectively).

2.9.3. DNA extraction

DNA from each cattle sample was extracted using the QIAGEN Gentra Puregene kit (PG) based on a non-membrane salting out method and two silica membrane-based

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protocols of the QIAGEN DNeasy Blood & Tissue kit: spin column (SC) and the 96-well plate (SP). DNA from each sheep sample was extracted using the PG and SC protocols.

According to the PG protocol, DNA is first isolated by removing red blood cells and lysing white blood cells. RNA and proteins are removed by enzyme digestion and salt precipitation, respectively. DNA is recovered by alcohol precipitation and dissolved in DNA hydration solution. The SC and SP protocols rely on a silica-based extraction method during which cells are lysed and transferred onto silica membranes to which DNA binds specifically during a centrifugation step. DNA is washed and finally eluted using a DNA hydration buffer. When possible, we performed different DNA extraction methods simultaneously on each sample. We followed the manufacturer's protocol with certain alterations to improve yield and quality of DNA samples. The most important alternation was that the silica protocols were started with a red blood cell lysis step that allowed us after centrifugation to transfer only the white blood cell pellet dissolved in PBS onto the silica membranes. This step removed impurities in the beginning of the protocol and improved purity measurements greatly. SC samples were also prepared in duplicates that were run through the same silica membrane to improve DNA yield. All alternations are detailed in S1 File. Fifteen cattle samples extracted by PG had to be re-purified following appendix C of the manufacturer's manual.

2.9.4. Quality control of DNA extracts

We employed a strict quality control (QC) strategy during DNA extraction and qPCR to ensure that samples extracted by different methods were of similar quality, purity and integrity. Our aim was to minimize the risk of differences between DNA extraction measurements being due to sample quality rather than differences of methods themselves. Samples failing QC were excluded from our final analyses (Table 1).

Table 1: Number of samples after each quality control step by species and method of DNA extraction. PG= Genra Puregene Kit; SC= Spin Column protocol (DNeasy Blood & Tissue Kit); SP= Spin Plate protocol (DNeasy Blood & Tissue Kit) * This step did not apply to SP. **Four samples were run on two qPCR plates only, because they did not yield enough DNA for more measurements.

Quality control step	DNA extraction method				
	Cattle			Sheep	
	PG	SC	SP	PG	SC
1. Starting samples	72	72	72	47	47
2. DNA yield >20ng/μl on Nanodrop	66	71	66	47	47
3. Protein contamination (260:280 ratio > 1.7)	66	71	66	47	41
4. Salt contamination (260:230 ratio > 1.8)	61	71	66*	47	39
5. DNA yield >20ng/μl on Qubit/FLUOstar	61	71	62	47	39
6. DNA integrity score <3	61	69	56	47	36
7. Sample selection (samples passing all tests for all methods)	56	56	51	36	36
8. Number of RTL measurements (sample number x qPCR plates)	224	224	196**	144	144
9. qPCR efficiencies for each triplicate within 5% of mean plate efficiency	224	223	196	144	144
10. Triplicate sample Cq values had CV < 5%	223	221	196	142	144

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We tested DNA yield and purity using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) with the software NanoDrop 2000. Samples with DNA concentrations less than 20 ng/ μ l were excluded from further investigation (Table 1). The average ratio of absorbance at 260 nm over 280 nm (OD 260/280) over two measurements was used to check for protein contamination and the average ratio at 260nm over 230nm (OD 260/230) was used to check for salt contamination. Both proteins and some salts can act as qPCR inhibitors (Demeke & Jenkins 2010). Extracts with OD 260/280 < 1.7 or OD 260/230 < 1.8 were excluded from further analyses for PG and SC methods. For SP, OD 260/230 readings were variable probably due to samples with low yields approaching the limit for accurate contaminant detection. We therefore decided not to exclude SP samples based on OD 260/230, although we applied the same OD 260/280 QC threshold as for the other methods. Note that results obtained from SP extracted samples behaved very similarly to the SC samples, despite the variable OD 260/230 ratios (see Results).

To assess DNA concentrations more accurately all PG and SC extracts were subsequently measured on a Qubit® 2.0 (Invitrogen) using a Qubit® dsDNA BR Assay kit (Invitrogen) according to the manufacturer's manual. SP extracts were measured on a FLUOstar Galaxy microplate reader (BMG LABTECH) using a Quant-iT™ dsDNA Assay Kit (Invitrogen) following the manufacturer's instructions. Both procedures are based on the detection of a fluorophore that becomes fluorescent when bound to double stranded DNA. Measurements are evaluated in relation to standards with known DNA concentrations. Because the signal is specific for double stranded DNA (dsDNA) fluorescence spectroscopy measurements are more accurate for DNA yield than NanoDrop measurements. Samples with average concentrations lower than 20 ng/ μ l calculated over two measurements on either fluorometer were excluded from further investigation. DNA integrity was assessed visually by running 200ng on a 0.5 % agarose gel with ethidium bromide at a final concentration of 0.8 μ g/ml. Gels were run at 100 mV and 200 mA for 45 minutes and then visualised with an Alphamager TM 2200. Gels were visually scored for integrity on a scale of 1 to 5 (Fig 1A) and extracts with a score greater than 2 were removed from further analyses. DNA stock solutions were prepared by diluting extracts to a concentration of 10 ng/ μ l based on fluorescence measurements. PG extracts were diluted in DNA hydration solution (QIAGEN), and SC and SP extracts were diluted in buffer AE (QIAGEN).

(A)

Gel image:					
Gel score:	1	2	3	4	5
Description:	<ol style="list-style-type: none"> 1. Clear DNA band 2. All DNA bands on the same level 3. no smear 	<ol style="list-style-type: none"> 1. Clear DNA band 2. All DNA bands on the same level 3. Smear visible but clearly distinct from DNA band 	<ol style="list-style-type: none"> 1. DNA band is not clearly distinct from smear 2. Majority of DNA on same level as DNA bands of samples with gel scores 1-2 	<ol style="list-style-type: none"> 1. No DNA band visible 2. All DNA further down the gel than DNA bands of samples with gel scores 1-2 	<ol style="list-style-type: none"> 1. DNA migrated very far along the gel 2. No DNA band visible

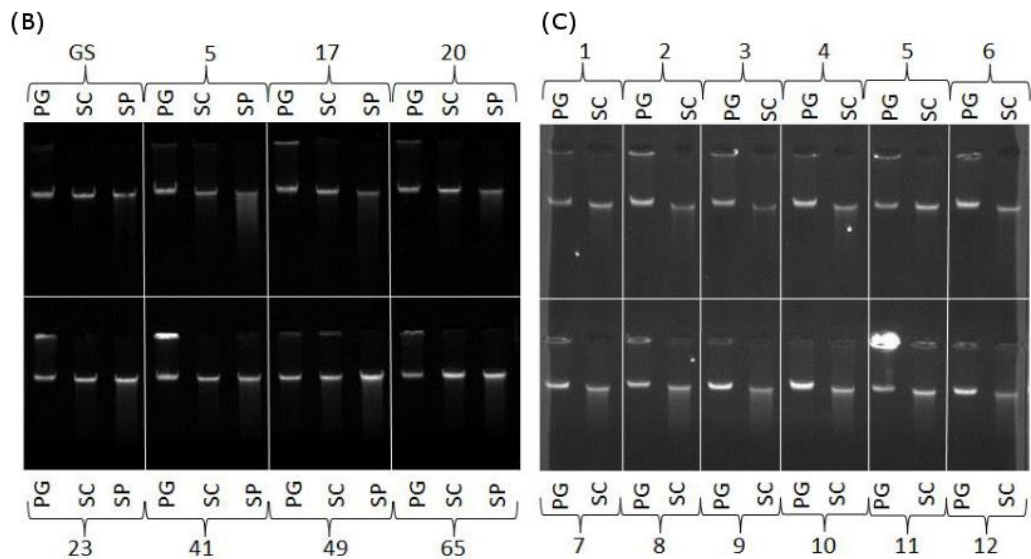


Figure 1: DNA integrity gels. (A) Illustrative DNA Integrity gels with gel scores. Example integrity gels for (B) Holstein Friesian cattle and (C) Soay sheep. Individual samples (represented by numbers in image) that were extracted with different DNA extraction protocols. (PG: Genra Puregene kit, SC: DNeasy spin columns, SP: DNeasy 96 well plate; GS: calibrator DNA (“golden sample”).

2.9.5. *Telomere length measurement*

Leukocyte RTL was measured by qPCR (Cawthon 2002) as the amount of telomeric DNA in a sample relative to the amount of a non-variable copy number reference gene. In order to identify the most appropriate reference gene we conducted preliminary analyses considering a variety of candidate reference gene primer pairs. The most consistent amplification profile and cleanest melting curve was obtained in both species using Primerdesign primers targeting the beta-2-microglobulin (B2M) gene (accession number: NM_001009284), which we selected as our reference gene. The selection of our reference gene was based on comparison of a panel of 12 candidate genes for sheep and 6 for cattle, supplied as part of the Primerdesign GeNorm kit (following Fairlie et al. 2016). B2M showed completely stable qPCR results indicative of non-variable copy number, and is well conserved and located on chromosome 10 of the bovine genome and chromosome 7 of the ovine genome (Ellis et al. 1993; Wu et al. 2008) . For the telomere amplification, tel 1b (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT) and tel 2b (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT) primers were used (Epel et al. 2004). Telomere primers were manufactured and purified with high performance liquid chromatography by Integrated DNA Technologies (IDT, Glasgow, UK).

The use of identical primers allowed us to use identical reaction conditions for both cattle and sheep qPCRs. We ran samples extracted by different methods and species on separate 384-well plates. Reactions for telomere and B2M primers were run in separate wells (monoplex qPCR) but on the same qPCR plate. Each qPCR plate was repeated four times over two days. Our calibrator sample came from a large volume of blood obtained from an individual cow or sheep, respectively. We extracted large quantities of DNA from each calibrator sample using different methods to match those applied to our experimental samples: PG, SC and SP for cattle, PG and SC for sheep. In the cattle experiment, each qPCR plate included three calibrator samples, one for each of the extraction methods used (i.e., calibrator samples extracted with PG, SC and SP methods). In the sheep experiment, we only included the MS calibrator on each plate (i.e. PG-extracted calibrator on plates of PG-extracted samples and SC-extracted calibrator on plates of SC-extracted samples).

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Samples and calibrators were loaded at a dilution of 1 ng/ μ l onto a 96 well plate (sample plate) that also contained a four step 1:4 serial dilution of calibrator DNA starting with 10 ng/ μ l as standard and nuclease free water as non-template control. A Freedom EVO 2150 robot (by TECAN) was used to transfer all samples, standards, calibrators and negative controls in triplicate onto a 384 well qPCR plate. The robot mixed 1 μ l of the contents of the sample plate with 9 μ l of master mix in each qPCR plate well. The master mix for both reactions contained 5 μ l of LightCycler 480 SYBR Green I Master (Roche) per well. Telomere primers were used at a concentration of 900 nmol, B2M primers were used at 300 nmol. Nuclease-free water was added to the master mix to have a final volume of 10 μ l per well.

The qPCR was performed on a LightCycler 480 (Roche) using the following protocol: Enzyme activation: 15 min at 95 °C; then 50 cycles of: 15 s at 95 °C (denaturation), 30s at 58 °C (primer annealing), 30 s at 72 °C (signal acquisition); melting curve: 1 min at 95 °C, 30s at 58 °C, then continuous increase of temperature (0.11 °C/s) to 95 °C with continuous signal acquisition; Cool down: 10 s at 40 °C. Melting curves showed a single peak with B2M primers rarely forming primer dimers in the negative controls. Telomere primers always form primer dimers due to the repetitive nature of their sequence. Evidence for primer dimer formation can be seen as melting peaks at slightly higher melting temperatures than the telomere qPCR product and also as amplification curves at very late cycles (average Cq for telomere negative controls: 38.1 (cattle) and 31.3 (sheep) compared to average Cq values of samples: 14.42 (SD = 0.76, cattle) and 13.52 (SD = 0.51, sheep)).

The software package LinReg PCR (Ruijter et al. 2009) was used to correct amplification curves for an estimated fluorescence baseline. The software also calculated well-specific amplification efficiencies. We used the mean efficiency across all wells on a plate, having excluded the upper and lower 5th percentiles, as our reaction efficiency for each amplicon group (Ruijter et al. 2009). The mean qPCR efficiencies across plates calculated with LinReg PCR ranged between 93.1%-94.2% (cattle) and 93.5%-94.0% (sheep) for the B2M reaction, and 93.6%-94.4% (cattle) and 92.5%-95.5% (sheep) for the telomere reaction. We set a constant fluorescence threshold within the window of linearity across all plates for the calculation of Cq values. The threshold was for B2M 0.221 in cattle and 0.1 in

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sheep and for the telomere amplification 0.256 and 0.1 in cattle and sheep, respectively.

We calculated mean qPCR efficiencies separately for both amplicon groups (B2M and telomere reaction) for each qPCR plate using LinReg PCR. Samples were excluded from final analysis if at least one of their triplicate amplifications had a qPCR efficiency that was 5% higher or lower than the mean efficiency for the respective amplicon. Also, samples were excluded if their Cq values had a coefficient of variation (CV) > 5% across triplicates. Elimination of samples that failed quality control for qPCR efficiency or Cq values ensured high intra-plate repeatabilities and efficiencies, although less than 1% of our samples were excluded based on these criteria (see Table 1).

RTL was calculated using following formula described by Pfaffl (2001):

$$RTL = \frac{E_{TEL}^{Cq_{TEL}(Calibrator)} - Cq_{TEL}(Sample)}}{E_{B2M}^{Cq_{B2M}(Calibrator)} - Cq_{B2M}(Sample)}}$$

where E_{TEL} and E_{B2M} are the reaction efficiencies for the plate for the respective amplicon group calculated by LinReg PCR; $Cq_{TEL[Calibrator]}$ and $Cq_{B2M[Calibrator]}$ are the mean Cq values across triplicates for the telomere and B2M reactions, respectively, for the plate's calibrator sample; and $Cq_{TEL[Sample]}$ and $Cq_{B2M[Sample]}$ are the mean Cq values across triplicates for telomere and B2M reactions, respectively, for the focal cattle or sheep sample.

An aim of our study was to test whether the use of a MS calibrator could control for differences in RTL amongst extraction methods. Therefore, in our initial cattle experiment we calculated RTL with the equation above but using three different calibrators: (1) a MS calibrator, (2) a calibrator extracted with a single method across all plates, arbitrarily choosing PG (termed "PG calibrator"), and (3) a constant Cq value across all plates ("no calibrator"). We chose constants of 26 for the reference gene and 14 for telomeres, as these were the average sample Cqs for these amplicons in our cattle experiment. The use of a constant Cq in the above equations allowed us to examine how well the use of a plate-specific calibrator (either MS or PG calibrators) accounted for plate-to-plate variation in RTL measures, whilst keeping RTL values on a similar scale as the RTLs calculated with

MS and PG calibrators. In the subsequent sheep experiment, we only compared the MS calibrator with the no calibrator calculations (25.99 for reference gene and 13.71 for telomeres). We also examined variation in the raw Cq values for the telomere and B2M reactions. It is important to note that higher Cq values represent lower concentrations of telomere or reference gene and vice-versa in our RTL calculations.

2.9.6. Statistical Analysis

Each sample was run on four identical qPCR plates per DNA extraction method. We calculated the Pearson's correlation coefficient for the individual RTL measurements between all possible plate combinations. We took the average RTL for a sample across the four plates within each extraction method and calculated the Pearson's correlation coefficient among methods. We calculated the CV– i.e. the standard deviation divided by the mean – across replicates of each sample both across all plates and within plate using the same extraction method. Pooled CVs across samples were calculated as the geometric mean CV.

Linear mixed models were used to estimate the repeatability of RTL measurements and Cq values for a given sample, the degree of plate to plate variation, and the effect of DNA extraction method on mean RTL. The model of analysis included the random effects of sample, sample-by-extraction method interaction and plate, and the fixed effect of DNA extraction method. Variance components for the random effects were estimated using restricted maximum likelihood. The sum of all variance components constituted the total phenotypic variance. The repeatability of sample RTL across plates and methods was calculated as the ratio of the sample variance to the total phenotypic variance. The ratio of the sample-by-extraction method interaction to total phenotypic variance provided an estimate of the proportion of variance attributable to differences in RTL among extraction methods within a sample, whereas the ratio of the plate effect to total phenotypic variance expressed the proportion of variance attributable to differences in the mean RTL among plates. We tested the significance of any differences in mean RTL associated with DNA extraction method by comparing models with and without extraction method as a fixed effect using a likelihood ratio test. We ran separate models for RTL calculated using MS calibrators (both species), PG calibrators (cattle only) and no calibrator (both species). We made the same comparisons for the reference gene and telomere Cq values in both species. All statistical analyses were performed in R

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Studio with R 3.1.2 (R Core Team 2014) with mixed-effects models being implemented using the 'lme4' library.

2.10. Results

2.10.1. DNA yield and integrity with different DNA extraction methods

A total of 56 of our PG and SC cattle samples, 51 of our SP cattle samples, and 36 of our sheep samples passed all quality controls for all DNA extraction methods and were used for RTL measurement (resulting in RTL measurements for a total of 235 DNA samples; Table 1). DNA yield was method dependent. The non-silica membrane-based PG extraction kit yielded the highest DNA concentrations (cattle: mean= 341 ± 6 ng/ μ l; sheep: mean= 282.6 ± 2 ng/ μ l) and highest total yields of DNA (cattle: mean = 76 ± 2 μ g; sheep: mean= 74 ± 1 μ g). The SC method produced substantially lower yields (cattle: mean concentration = 120 ± 2 ng/ μ l, mean total yield = 12 ± 0.2 μ g; sheep: mean concentration= 68 ± 1 ng/ μ l, mean total yield= 15 ± 0.2 μ g) and the SP method lower still (cattle: mean concentration = 38 ± 0.6 ng/ μ l; mean total yield = 3 ± 0.05 μ g). However, initial whole blood volumes of cattle varied between DNA extraction methods (PG: 3 ml, SC: 600 μ l, SP: 300 μ l), whereas the same volumes of sheep buffy coat were used in all cases.

We also noticed that DNA integrity gels varied in appearance across extraction methods (Fig 1B). PG extracts showed the cleanest bands with no signs of smears and thus no signs of DNA disintegration. Based on our numeric integrity gel score (Fig 1A) all PG samples for both species scored a 1 (best score) while all spin column samples for sheep and 2 out of 69 samples for cattle scored a 2. Of the SP samples the majority of samples (83.9%) passed with a gel score of 2. A total of 11 SC or SP extracts from both species failed quality control based on their integrity gel score (Table 1).

2.10.2. Repeatability of telomere length measurements & effects of DNA extraction method

We found relatively high correlation coefficients and low CVs across plates for RTL measurements of the same sample in both species. All correlation estimates both within DNA extraction method (across plates) and between methods for the two species are summarized in S2 File. Correlations among RTL measurements from the same sample, calculated using a MS calibrator, among plates ranged from 0.87 to 0.96 for cattle, and 0.83 to 0.93 for sheep (S2 File). Correlations between average

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RTL measurements derived from different extraction methods and using different calibrators are summarised in Fig 2. Using a MS calibrator, correlations between the PG and SC methods were 0.85 for cattle and 0.77 for sheep, whilst in cattle the correlation between PG and SP was 0.78 and between SC and SP 0.87 (Fig 2). The correlation coefficients were comparable when a PG calibrator or no calibrator was used for RTL calculation (Fig 2). However, when fitting regression lines among samples extracted using different methods, application of the MS calibrator clearly produces regression slopes much closer to one with intercepts close to the origin (Fig 2). The average CV across all plates was 8.2% in cattle (12 plates, 3 methods), and 8.1% in sheep (8 plates, 2 methods). Within extraction method, CVs across plates were 9.2% and 8.2% for PG, 5.1% and 4.5% for SC, for cattle and sheep, respectively, and 5.2 % for the SP in cattle only.

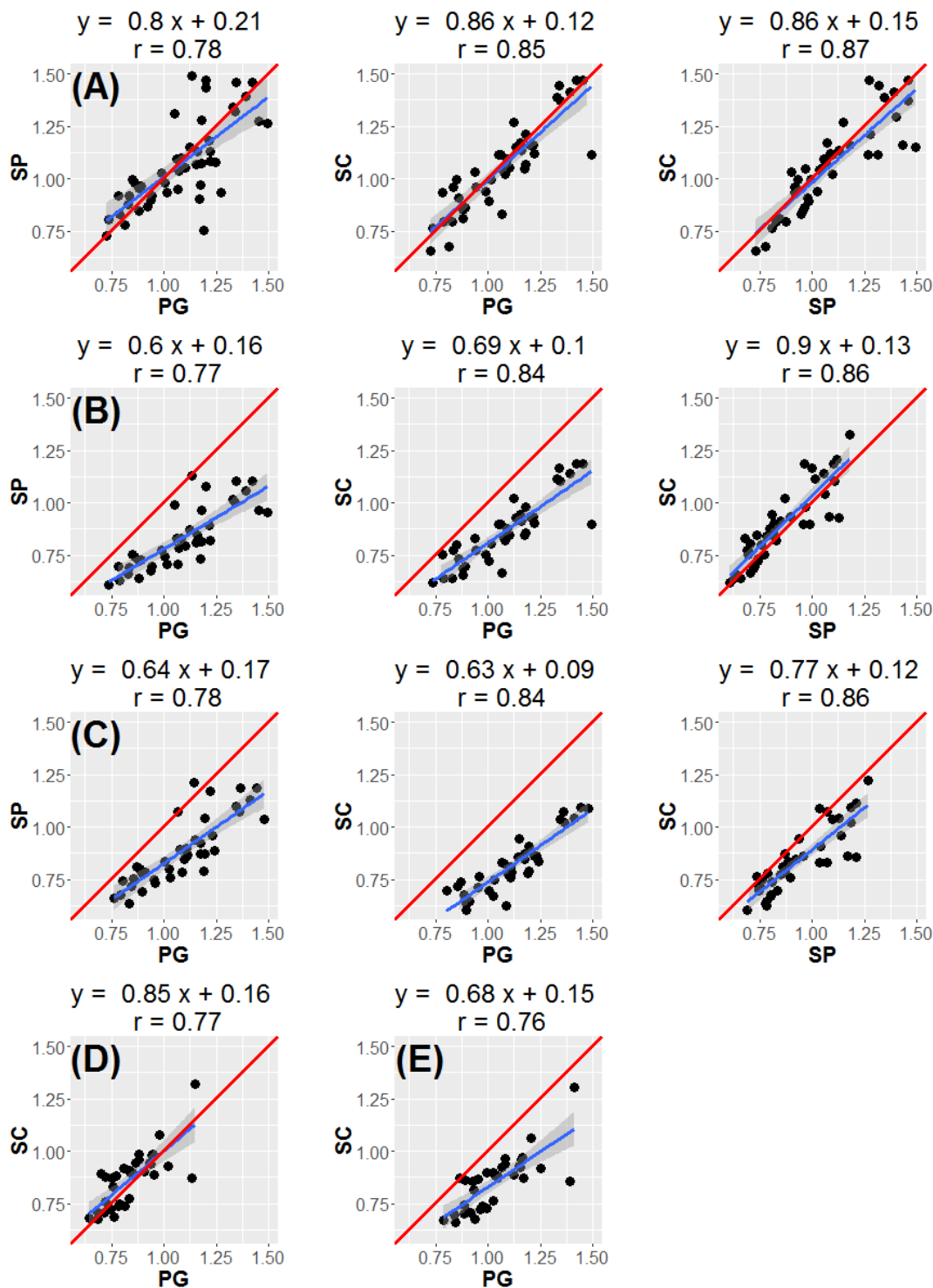


Figure 2: Correlations between methods. Correlations between RTL measurements from different DNA extraction methods (PG: Gentra Puregene kit; SC: DNeasy spin columns; SP: DNeasy 96 well plate): Cattle, method-specific calibrator (A); Cattle, Puregene calibrator (B); Cattle, no calibrator (C); Sheep, method-specific calibrator (D); Sheep, no calibrator (E). Regression lines and their 95% confidence interval are shown in blue and grey, respectively, with red lines reflecting a hypothetically perfect correspondence (slope of one, intercept of zero).

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In both cattle and sheep, we found significantly ($P < 0.05$) higher mean RTL in samples extracted using the non-membrane-based method (PG) compared to those extracted with the silica membrane-based methods (SC and SP), when using either the PG calibrator or no calibrator in calculations (Fig 3, Table 2). This reflects genuine underlying differences in the average TL among DNA extracted from the same sample by different methods, as has been reported elsewhere (Cunningham et al. 2013; Hofmann et al. 2014). These differences are underpinned by either or both lower telomeric Cq and higher reference gene Cq values in the PG extracted samples compared to the other methods (Fig 3D, E, H & I). In both species, there was notable variation in the telomeric Cq values across plates run on the same day, with the first plate having lower values than the second (Fig 3D & H). As would be expected, application of a plate-specific calibrator (either PG or MS calibrators) removed the within-day variation in RTL and substantially reduced among-plate variation (Table 2; Fig 3). Importantly, the differences in mean RTL among extraction methods became non-significant and sample repeatabilities were increased when a MS calibrator was used to calculate RTL (Fig 3, Table 2). This shows that using a MS calibrator to calculate RTL can account for observed effects of DNA extraction method on the underlying Cq values (Table 2; Fig 3).

Table 2: Variance component and parameter estimates. MS calibrator: Method specific calibrator, PG calibrator: Puregene extracted calibrator.

	σ^2 Total	σ^2 Sample	σ^2 Sample x Method	σ^2 Plate	σ^2 Residual	r^2 Sample	r^2 Sample x Method	r^2 Plate	X^2 Method	P Method	Effect PG vs. SC \pm SE	Effect PG vs. SP \pm SE
Cattle												
RTL calculated with MS calibrator	0.053	0.038	0.007	0.003	0.005	0.717	0.130	0.064	0.710	0.701	-0.035 \pm 0.045	-0.016 \pm 0.045
RTL calculated with PG calibrator	0.041	0.028	0.006	0.004	0.004	0.671	0.137	0.098	20.434	<0.001	-0.244 \pm 0.048	-0.283 \pm 0.048
RTL calculated with no calibrator	0.057	0.028	0.006	0.020	0.004	0.482	0.103	0.354	9.537	0.008	-0.330 \pm 0.102	-0.237 \pm 0.102
Cq for <i>B2M</i> (reference gene)	0.293	0.126	0.155	0.004	0.008	0.430	0.528	0.015	31.069	<0.001	-0.138 \pm 0.089	-0.638 \pm 0.091
Cq for telomere amplification	0.507	0.296	0.140	0.054	0.017	0.585	0.276	0.106	11.407	0.003	0.409 \pm 0.179	-0.271 \pm 0.180
Sheep												
RTL calculated with MS calibrator	0.020	0.012	0.003	0.002	0.002	0.615	0.162	0.113	0.620	0.431	0.027 \pm 0.036	/
RTL calculated with no calibrator	0.034	0.014	0.004	0.012	0.003	0.430	0.113	0.359	4.815	0.028	-0.179 \pm 0.079	/
Cq for <i>B2M</i> (reference gene)	0.150	0.051	0.083	0.013	0.003	0.338	0.555	0.084	11.185	0.001	-0.414 \pm 0.105	/
Cq for telomere amplification	0.238	0.108	0.099	0.026	0.006	0.452	0.417	0.108	0.498	0.481	-0.091 \pm 0.136	/

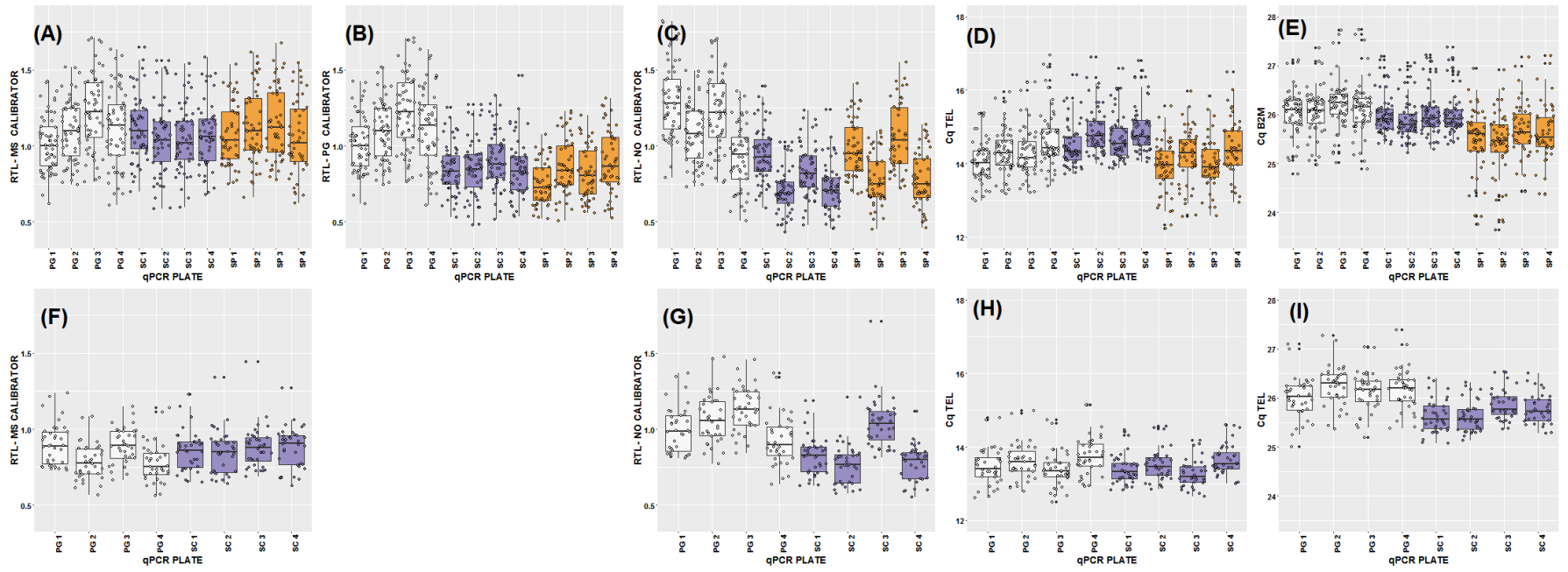


Figure 3: Raw RTL and Cq values. RTL or Cq values by DNA extraction method and qPCR plate for cattle (A-E) and sheep (F-I). RTL calculated with method specific (MS) calibrator (A + F), Puregene (PG) calibrator (B), no calibrator (C+G). Cq values for telomere reaction (D+H) and control gene B2M (E+I). Colours represent DNA extraction methods. White: Gentra Puregene, blue: DNeasy spin columns, orange: DNeasy 96 well plate.

2.11. Discussion

In the present study, we addressed the effect of DNA extraction method on RTL measurements by comparing two silica membrane-based kits (SC and SP) with a kit that uses a non-membrane-based salting out method (PG). As expected (Psifidi et al. 2015), we found that the salting out method produced higher DNA yields and that silica membrane-based methods were associated with some observable loss of DNA integrity (Fig 1). A number of studies using human blood samples report significant differences in mean RTL depending on the DNA extraction method used (Cunningham et al. 2013; Hofmann et al. 2014; Denham et al. 2014; Tolios et al. 2015; Raschenberger et al. 2016). We found that silica membrane-based DNA extraction methods produced shorter RTL measurements on average than the salting out method in both cattle and sheep. This is consistent with two previous studies in humans, which argued that silica membrane based DNA extraction methods reduce average RTL (Cunningham et al. 2013; Hofmann et al. 2014). However, the physical and biochemical causes of these observed extraction method effects on RTL measurements are currently unknown, and determining these causes is an important next step for research in this area.

We found that the rank order of RTL measurements among samples is largely unaffected by DNA extraction methods. Across extraction methods, our RTL measures showed reasonably high repeatabilities and inter-plate correlations and low inter-plate CVs that were close to those reported in the qPCR telomere literature (Aviv et al. 2011; Raschenberger et al. 2016; Cawthon 2002). The aforementioned studies on human samples do not offer clear insight into how extraction methods affect the rank order of RTL measurements. One study reported relatively high correlations among samples extracted by QIAmp spin columns (QIAGEN) versus a magnetic bead extraction (Spearman's $\rho = 0.71$) (Hofmann et al. 2014); another study found a moderate correlation between a magnetic bead and a salting out extraction (Pearson's $r = 0.54$) (Raschenberger et al. 2016). A third study found very low and not statistically significant correlations ($r < 0.21$) (Denham et al. 2014), and two of the studies did not present among sample correlations (Cunningham et al. 2013; Tolios et al. 2015). The absence of a strong correlation among RTL measurements based on different DNA extraction methods is a profoundly alarming result for research on telomere dynamics. If rank order of RTL is generally altered by underlying aspects of sample preservation, then associations among RTL and

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environmental, genetic and health measures within studies could themselves depend on the extraction method used. However, the one study reporting low correlations among RTLs based on different extraction methods used DNA samples that would have failed our QC criteria (Denham et al. 2014) and it seems likely that the low correlations may be the result of variation in the level of DNA impurities that might have acted as qPCR inhibitors. Our results show that, as long as rigorous QC criteria are applied throughout telomere measurement protocols, the rank order of samples is very largely preserved regardless of the DNA extraction method used, despite the distribution of RTL estimates changing (Fig 2). Failure to carefully monitor and control the integrity and purity of DNA is likely to result in increased sampling error which will reduce the repeatability of results both within and among studies of telomere dynamics.

Importantly, our results show that it is possible to account for differences in mean RTL associated with DNA extraction method using a DNA extraction method-specific calibrator. Our reading of the literature suggests it is unusual for qPCR-based telomere studies in both epidemiology and ecology to provide much information about the source or preparation of the calibrator sample used. The five previous studies of DNA extraction method effects on RTL discussed above presumably used a calibrator sample extracted using only one extraction method, although most of them fail to explicitly state what kind of calibrator was used (Cunningham et al. 2013; Hofmann et al. 2014; Tolios et al. 2015; Denham et al. 2014) and how it was extracted (Cunningham et al. 2013; Hofmann et al. 2014; Tolios et al. 2015; Denham et al. 2014; Raschenberger et al. 2016). This is entirely reasonable given the aim was to test for differences in the telomere to control gene ratios associated with DNA extraction method. In this study, we have demonstrated a relatively simple approach that could account for DNA extraction method effects on RTL that could potentially allow researchers to perform qPCR based telomere studies combining samples extracted in different ways. By extracting large quantities of DNA from a single large sample of blood by different methodologies and running these on appropriate plates, we were able to apply an extraction method-specific calibrator in our calculations of RTL. This accounted for the extraction method effects on mean RTL which were observed in our two data sets when the standard calibration approach was used. More generally, our data suggest that within qPCR-based studies of TL, calibrator samples could be used for more than just accounting for plate to plate variation. As long as DNA integrity and purity is carefully controlled,

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calibrator samples derived from the same original sample but collected, stored or extracted in different ways could conceivably be used to control for systematic effects of variation in sample preparation on RTL.

It is obviously preferable to use a completely consistent approach and extract DNA using the same method within a study. However, a major challenge in the study of telomere dynamics is to generate sufficiently detailed longitudinal data to determine whether variation in TL observed later in life is the result of differences set in early life or differences in attrition rates across life (Benetos et al. 2013). Addressing this challenge in long-lived animals will inevitably require the use of long-term longitudinal archived samples, in which samples may have been stored or DNA extracted in different ways over time. Our calibrator-based approach could allow such valuable longitudinal samples to be compared within a single study, but it would need to be carefully validated each time it was applied. We would advocate applying similarly stringent quality control on DNA integrity and purity as here, even though this may reduce the available sample size. Before applying a method-specific calibrator approach to archived samples prepared in different ways, it would also be crucial to run a similar experiment to establish the repeatability of RTL measures among samples that have been experimentally exposed to the relevant differences in sample collection, storage or DNA extraction.

2.12. Conclusion

This study adds to the emerging literature showing that DNA extraction methods may affect the mean RTL measurement produced by qPCR techniques. We present the first evidence for such effects in non-human vertebrates, documenting similar results in two ruminant species of considerable economic and agricultural importance in which TL variation has recently been examined with some exciting initial results (Brown et al. 2012; Fairlie et al. 2015; Laubenthal et al. 2016). We also show that RTL measurements derived from different DNA extraction methods are highly correlated when rigorous DNA quality control is applied. Our results also suggest that the application of method-specific calibration in qPCR studies of RTL could allow researchers to effectively use valuable historical archives of samples that have been prepared or extracted in different ways, accounting for effects of methodological variation on mean RTL.

2.13. Acknowledgements

We thank everyone involved in collecting blood samples on the Crichton farm, particularly Hannah Dykes and Isla McCubbin. We are grateful to Josephine Pemberton, Jill Pilkington, the 2013 August Soay sheep catch volunteers, and the SynthSys facility at the University of Edinburgh for support with telomere measurements. We also thank Ian Archibald for support with data handling, and Jarrod Hadfield for helpful discussions and statistical advice.

2.14. Thesis context

In the present chapter robust methods for the measurement of RLTL in cattle are presented, thereby addressing the first objective of the thesis. It is demonstrated that qPCR based TL measurements are highly repeatable even if samples are extracted repeatedly and even if different DNA extraction methods are used. The effect of different DNA extraction methods is tested to investigate if fast silica based methods lead to shorter RLTL measurements. A simple method is presented that corrects for DNA extraction method effects. It is shown that silica based extracts lead to more repeatable RLTL measurements with qPCR. Furthermore, silica based methods are faster, cheaper and cleaner; therefore, they are the best candidate method for large-scale telomere projects. This method was used to measure TL of all animals and samples in the rest of this thesis.

2.15. Chapter appendix 1 (Supplementary File 1): Protocols for the extraction of DNA from bovine whole blood and ovine buffy coat

2.15.1. DNA Extraction from bovine whole blood using salting out method (Gentra Puregene kit by QIAGEN)

1. **Thaw** whole blood samples slowly at **4 °C** (takes for 1-5 ml approximately 2-3 hours).

(N.B. The manufacturer's manual recommends thawing quickly in the water bath at 37 °C, but slow thawing might help if DNA yields tend to be low.)

2. Dispense **9 ml RBC Lysis Solution** into a 15 ml centrifuge tube.
3. Add **3 ml whole blood** and mix by **inverting 10 times**.
4. Incubate **5 min at room temperature** (15-25°C). **Invert** at least once during incubation.
5. Centrifuge for **2 min at 2000 x g** to pellet the white blood cells.
6. **CAREFULLY pipette off supernatant** (10 ml pipette, then 1 ml pipette) leaving approximately **200 µl of the residual liquid** and the white blood cell pellet.
7. Vortex the tube vigorously to re-suspend the pellet in the residual liquid. *Vortexing greatly facilitates cell lysis in the next step. The pellet should be completely dispersed after vortexing.*
8. Add **3 ml Cell Lysis Solution** and **pipet up** and down to lyse cells. **Vortex vigorously for 10 s**.

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Samples are very stable at that stage (up to 2 years at room temperature) and can be left in the fridge overnight.

9. Set **water bath to 37 °C**.
10. Add **15 µl RNase A Solution (QIAGEN)** and mix by **inverting 25 times**.
Incubate for 15 min at 37 °C.
11. Incubate for **3 min on ice** to quickly cool down the samples.
12. Set **water bath to 65 °C**.
13. Add **1 ml of Protein Precipitation Solution**, and **vortex vigorously for 20 s** at high speed.
14. **Centrifuge for 5 min at 2,000 x g**.
The precipitated protein should form a tight, dark brown pellet. If not, pipet up and down, incubate sample on ice for 5 min and repeat centrifugation.
15. Pipet **3 ml isopropanol into a clean 15 ml tube** and **add the supernatant** from the previous step by pipetting (10 ml pipette).
Be sure the protein pellet is not dislodged.
16. **Mix by inverting gently 50 times** until the DNA is visible as threads or a clump.
17. **Transfer DNA and 1 ml of liquid to a 1.5 ml micro-centrifuge tube** (use 1 ml pipette with broad tips)

(N.B. This is the main alternation of the original protocol where the DNA pellet stays in 15 ml falcon tubes during all washing and elution steps. We found that it is less likely to lose DNA if the pellet is transferred to a micro-centrifuge tube and if the micro-centrifuge protocol provided by the manufacturer is followed until DNA elution.)

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18. Centrifuge **1 min at 13,000 x g**.
19. **Pipette off isopropanol** (centrifuge again and pipette off residual).
20. Wash with **500 µl 70 % Ethanol**.
21. Centrifuge **1 min at 13,000 x g, pipette off Ethanol**, centrifuge again and pipette off residual).
22. **Dry until pellet gets a bit translucent** (~2-8 min).
23. Add **DNA hydration solution** to dissolve gDNA (50 – 400 µl depending on pellet size).
24. Incubate at **65 °C for 1 hour** to dissolve gDNA.
25. Incubate at **room temperature overnight** on **rotator**.
26. Measure DNA yield and quality (260/280, 260/230) on the next day (NanoDrop).
27. Samples are stable at 4 °C indefinitely but for long term storage put at – 20 °C.

(Protocol can be started with step 9 for samples that have been stored overnight in Cell Lysis Solution. During the following steps the next lot of samples can thaw in the fridge. They can be prepared until step 8 during step 24.)

2.10.2. DNA Extraction from bovine whole blood using silica membrane based extractions (DNeasy Blood & Tissue Kit by QIAGEN)

2.10.2.1. Spin column protocol

- Step 1.-12. are prepared in duplicates to increase DNA yield. Therefore, do step 14. with the first duplicate, place spin column in a new 2 ml micro centrifuge

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tube, add second duplicate and centrifuge again for 1 min at 8,800 rpm. Discard flow through and collection tube and continue with step 15.

1. **Thaw** whole blood samples slowly at **4 °C** (takes for 1-5 ml approximately 2-3 hours).

(N.B. The manufacturer's manual recommends thawing quickly in the water bath at 37 °C, but slow thawing might help if DNA yields tend to be low.)

2. Dispense **900 µl RBC Lysis Solution** into a 2 ml micro-centrifuge tube.

(N.B. We added this additional step similar to the beginning of the Puregene extraction protocol to the beginning of the DNeasy extractions where we lysed and removed red blood cells, keeping only the leukocyte pellet for the subsequent DNA extraction. Before, a low OD 260/230 ratio implied salt contamination for which EDTA could have been the reason. By removing a majority of protein and salts before samples were applied to silica membranes, both NanoDrop ratios for purity measurement improved notably indicating an improvement in DNA purity due to the changes of the protocol. We added steps 1.-7. to the manufacturer's protocol.)

3. Add **300 µl whole blood** and mix by **inverting 10 times**.
4. Incubate **5 min at room temperature** (15-25°C). **Invert** at least once during incubation.
5. Centrifuge for **30 s at 14,000 x g** to pellet the white blood cells.
6. **CAREFULLY pipette off supernatant** (1 ml pipette) leaving approximately **10 µl of the residual liquid** and the white blood cell pellet.

Vortex the tube vigorously to resuspend the pellet in the residual liquid.

Vortexing greatly facilitates cell lysis in the following steps. The pellet should be completely dispersed after vortexing.

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7. Dispense cell pellet in **90 µl PBS** by pipetting up and down and vortexing.

8. Add **30 µl Proteinase K**.

(N.B. We increased the volume of Proteinase K from 20 to 30 µl to improve purity of the extracts further.)

9. Add **4 µl RNase A** (100 mg/ml) and incubate for 2 min at room temperature.

10. Add **300 µl Buffer AL** (without added ethanol). Mix thoroughly by vortexing, and incubate at **56 °C for 10 min**.

Ensure that ethanol has not been added to Buffer AL (see “Buffer AL”, page 18). Buffer AL can be purchased separately (see page 56 of the manufacturer’s hand book for ordering information).

It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution

(N.B. Instead of 200 µl we used 300 µl buffer AL to promote cell Lysis. For the same reason we repeated the following incubation step at 56 °C after vortexing the samples thoroughly in step 38.)

11. Vortex samples for **10 s** and incubate for **10 more minutes at 56 °C**.

12. Add **200 µl ethanol (96–100%)** to the sample, and mix thoroughly by vortexing.

It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution

13. Place **AE buffer** in the water bath at **56 °C**.

14. Pipet the mixture from step 12 into the **DNeasy Mini spin column placed in a 2 ml collection tube** (provided). Centrifuge at **8,800 rpm for 1 min**.

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Discard flow-through and collection tube.¹ (If duplicates were prepared, repeat this step with the second duplicate on the same spin column.)

15. Place the DNeasy Mini spin column in a new 2 ml microcentrifuge tube (not provided), add **300 µl Buffer AW1**, and wash the inside of the DNeasy Mini spin column thoroughly by **inverting each tube 5 times and then roll them in your fingers 5 times**.

Centrifuge for **1 min at 8,800 rpm**. Discard flow-through and collection tube.¹

(N.B. Both washing steps with buffer AW1 and AW2 were performed with 300 µl buffer (instead of 500 µl), but they were repeated once each. Rolling and inverting the tubes containing the washing buffer ensured that the inside of the tubes including the lids were reached and cleaned by the buffers.)

16. **Repeat step 15.**

17. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add **300 µl Buffer AW2**, and wash the inside of the DNeasy Mini spin column thoroughly by **inverting each tube 5 times and then roll them in your fingers 5 times**.

Centrifuge for **3 min at 14,000 rpm (20,000 x g)** to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

18. **Repeat step 17.**

19. *Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-*

¹ Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 of the DNeasy handbook for safety information.

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through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

20. Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and pipet **100 µl Buffer AE (warm; see step 13) directly onto the DNeasy membrane.**

(N.B. To increase the elution efficiency the elution buffer AE was warmed to 56°C before 100 µl were added onto the silica membranes. Samples were then incubated for 10 min at 56°C in a heat block before final centrifugation for elution.)

21. Incubate at **56 °C for 10 min** (use heat block to prevent condensation water in the lids), and then centrifuge for **1 min at 8,800 rpm** to elute.
22. If samples were prepared in duplicates, do two elution steps: Pipet **50 µl warm Buffer AE** directly onto the DNeasy membrane, incubate at **56°C for 5 min** (use heat block) and centrifuge for **1 min at 8,800 rpm.**

If you want to use more than 200 µl buffer AE in total to elute the DNA of a single sample, use a new microcentrifuge tube for the second elution step. Otherwise, the silica membrane will touch the flow through.

2.10.2.2. Spin plate protocol

N.B. Red blood cells are lysed and white blood cells are spun down and collected for DNA extraction prior to the beginning of the manufacturer's protocol. Because preparation of samples in this way was not successful in deep 96 well plates, samples are prepared in micro-centrifuge tubes until they are transferred onto silica membranes. From this step onwards the manufacturer's protocol is followed with minor alternations:

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1. **Thaw** whole blood samples slowly at **4 °C** (takes for 1-5 ml approximately 2-3 hours; can be done overnight).
2. Dispense **900 µl RBC Lysis Solution** into a 1.5 ml micro-centrifuge tube (not provided by the kit).
3. Add **300 µl whole blood, seal plate** and mix by **inverting 10 times**.
4. Incubate **5 min at room temperature** (15-25 °C). **Invert** at least once during incubation.
5. Centrifuge for **30 s at 14,000 x g** to pellet the white blood cells.
6. **CAREFULLY pipette off supernatant** (1 ml pipette) leaving approximately **10 µl of the residual liquid** and the white blood cell pellet.
Vortex vigorously to re-suspend pellet in residual liquid.

Vortexing greatly facilitates cell lysis in the following steps. The pellet should be completely dispersed after vortexing.
7. **Re-suspend white blood cell pellet in 190 µl PBS and transfer.**
8. Add **20 µl proteinase K** to each sample and mix thoroughly.
9. Add **4 µl RNase A** (100 mg/ml) and incubate for **5 min at room temperature**.
10. Add **200 µl Buffer AL** (without added ethanol) to each sample and mix by pipetting up and down.
11. Vortex **vigorously for 15 s**.
12. Incubate at **56°C for 10 min**. Mix occasionally during incubation to disperse the sample, or place on a rocking platform.

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13. Add **200 µl ethanol (96–100%)** to each sample.
14. Vortex **vigorously for 15 s**.
15. **Place two DNeasy 96 plates on top of S-Blocks** (provided by kit). Mark the DNeasy 96 plates for later sample identification
16. **Carefully transfer the lysis mixture (maximum 900 µl) of each sample from step 16 to each well of the DNeasy 96 plates.** Take care not to wet the rims of the wells to avoid aerosols during centrifugation. Do not transfer more than 900 µl per well.

Note:

Lowering pipet tips to the bottoms of the wells may cause sample overflow and cross-contamination. Therefore, remove one set of caps at a time, and begin drawing up the samples as soon as the pipet tips contact the liquid. Repeat until all the samples have been transferred to the DNeasy 96 plates.

17. **Seal each DNeasy 96 plate with an AirPore Tape Sheet** (provided by kit). **Centrifuge for 4 min at 6,000 rpm.**

AirPore Tape prevents cross-contamination between samples during centrifugation. After centrifugation, check that all of the lysate has passed through the membrane in each well of the DNeasy 96 plates. If lysate remains in any of the wells, centrifuge for a further 4 min.

18. Remove the tape. Carefully **add 500 µl Buffer AW1** to each sample.

Note:

Ensure that ethanol has been added to Buffer AW1 prior to use.

19. **Seal each DNeasy 96 plate with a new AirPore Tape Sheet** (provided by kit). **Centrifuge for 2 min at 6,000 rpm (=5796 x g).**

20. **Repeat steps 18-19.**

21. Remove the tape. Carefully **add 500 µl Buffer AW2** to each sample.

Note:

Ensure that ethanol has been added to Buffer AW2 prior to use.

22. Place **buffer AE** in the water bath at **56°C**.

23. **Centrifuge DNeasy Plates for 15 min at 6,000 rpm.**

Do not seal the plate with AirPore Tape.

The heat generated during centrifugation ensures evaporation of residual ethanol in the sample (from Buffer AW2) that might otherwise inhibit downstream reactions.

24. Place each DNeasy 96 plate in the correct orientation on a **new rack of Elution Microtubes RS** (provided by kit).

25. To elute the DNA, add **80 µl Buffer AE** (warm; see step 24) to each sample, and seal the DNeasy 96 plates with new AirPore Tape Sheets (provided by kit).

Incubate for 1 min at room temperature (15–25°C) (or better **for 10 min at 56°C**). **Centrifuge for 4 min at 6,000 rpm.**

(200 µl Buffer AE is sufficient to elute up to 75% of the DNA from each well of the DNeasy 96 plate. Elution with volumes less than 200 µl significantly increases the final DNA concentration of the eluate but may reduce overall DNA yield. For samples containing less than 1 µg DNA, elution in 50 µl Buffer AE is recommended.)

26. **Recommended:** For maximum DNA yield, repeat step 27 with another 80 μ l Buffer AE.

(A second elution with Buffer AE will increase the total DNA yield by up to 25%. However due to the increased volume, the DNA concentration is reduced. If a higher DNA concentration is desired, the second elution step can be performed using the 200 μ l eluate from the first elution. This will increase the yield by up to 15%. Use new caps (provided) to seal the Elution Microtubes RS for storage.)

2.10.3. Extraction from ovine buffy coat using Genra Puregene Kit (QIAGEN)

Most alterations were done to allow working with Eppendorf tubes rather than with 15 ml falcon tubes. For being able to prepare samples in tubes that are so much smaller, volumes of some reagents had to be adjusted. Also, centrifugation times were adapted to micro- centrifuges.

1. Thaw buffy coat quickly in 37°C water bath, store on ice. (change WB temp to 55 °C)
2. Aliquot 100 μ l buffy coat into 2ml Eppendorf micro-centrifuge tube. Return remaining stock buffy to correct box in -20 °C, having marked to indicate that it has been thawed.

Because of high DNA yields extractions were started with less buffy coat than recommended in the manufacturer's manual (150-200 μ l).

3. Add 600 μ l RBC lysis solution, pipette up and down to mix.

Red blood cells were lysed by adding 600 μ l instead of 300 μ l red blood cell lysis solution to ensure lysis of red blood cells.

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4. Incubate for 10 min at room temperature with mixing if there are lumps.
5. Centrifuge 3 min at 13,000 x g.
6. Pipette off supernatant leaving ~ 150 μ l. Re-suspend pellet until no lumps are visible by tube flicking and vortexing. Avoid using a pipette, to prevent losing material in tip.
7. Add 1ml cell lysis solution, pipette and vortex to mix.
8. Incubate 1 hour at 55 °C to dissolve all lumps. If still viscous and lumpy, add 1-2 μ l proteinase K to further digest.
9. Add 10 μ l RNase solution, mix by inverting. Incubate 37 °C for 15 min.
10. Chill on ice 3 min.
11. Add 400 μ l protein precipitation solution and vortex for 20 s at high speed.
12. Centrifuge 3 min at 13,000 g
13. Pipette supernatant into 2 x 500 μ l isopropanol. Invert until strings of gDNA are visible. *The manufacturer's protocol recommends using 3 ml of Isopropanol in a 15 ml falcon tube, but we found that 1 ml is sufficient to pellet the DNA and that working with Eppendorf tubes reduces the risk to lose the DNA pellet.*
14. Centrifuge 1 min 13,000g. Use pipette to remove excess isopropanol.

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15. Wash with 400 μ l 70 % ethanol.
16. Centrifuge 1min 13,000g, pipette off ethanol. Leave upturned on paper towel on bench to dry for 4-5 min, or until pellet is translucent.
17. Add DNA hydration solution to dissolve gDNA (100-300 μ l depending on pellet size), then incubate at 65 °C 1 hour to dissolve gDNA.
18. Leave overnight on rotator and measure on Nanodrop the next day. Samples are then stable 4 °C indefinitely but store long-term at -20 °C.

2.16. Chapter appendix 2 (Supplementary File 2): Correlation Matrices

Correlation matrices within (across plate) and between DNA extraction methods

Explanation of axis labels:

SP	Spin Plate extracts (qPCR plates 1-4)
PG	Puregene extracts (qPCR plates 1-4)
SC	Spin Column extracts (qPCR plates 1-4)
MS.cal	Method-specific calibrator
PG.cal	Puregene calibrator
No.cal	No calibrator
average	Average RTL values calculated across 4 plate repeats

The colour bar underlying each plot explains the colour coding that was applied to visualise the strength and direction of individual correlations. No correlation would appear as white field, negative correlations as different shades of red and positive correlations as different shades of blue. It can be seen very quickly that all correlations are positive and high.

N.B. Correlation coefficients do not exactly agree with figure 2, because only complete observations (without missing values) can be used for the calculation of correlation matrices. Therefore, for cattle they are based on 44 samples, whereas correlation coefficients shown in figure 2 are based on up to 56 samples. For sheep correlation matrices were calculated using 35 complete observations (out of 42).

2.16.1. Cattle

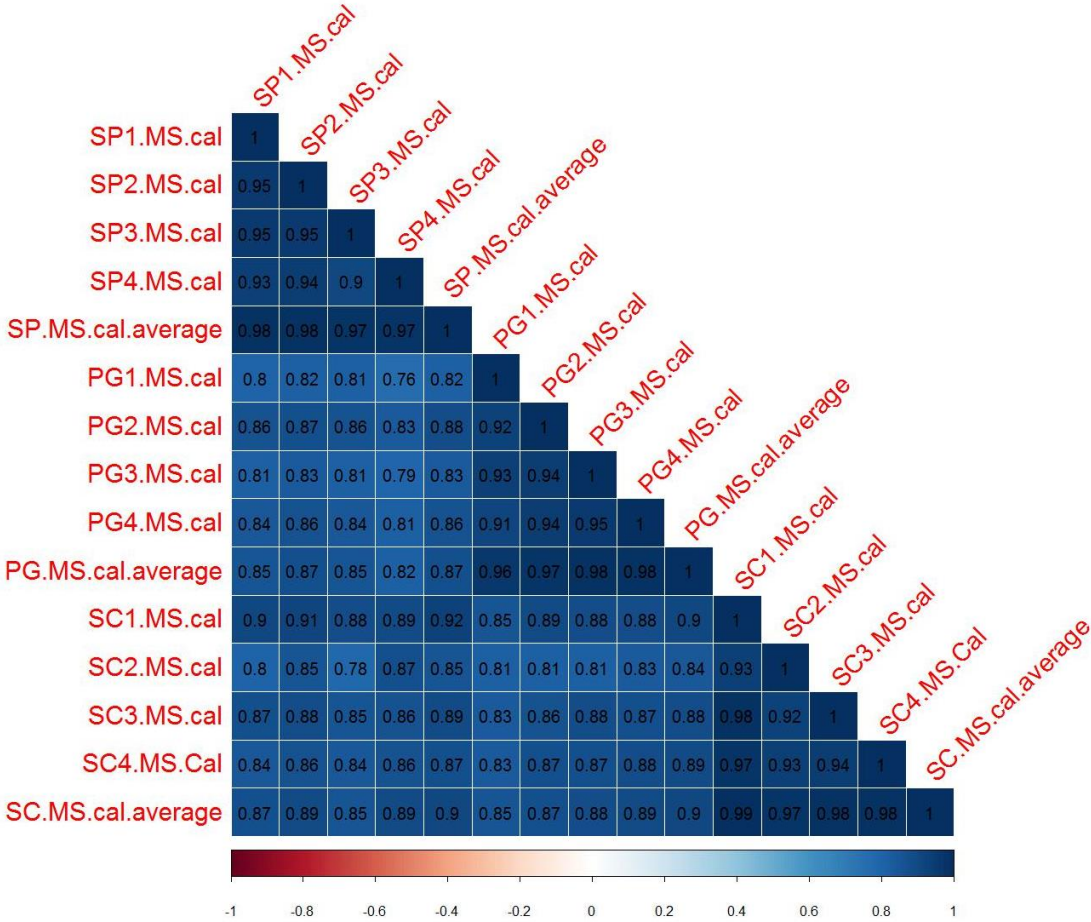


Figure S1: Cattle: Correlations among RTL measurements calculated using a method-specific calibrator

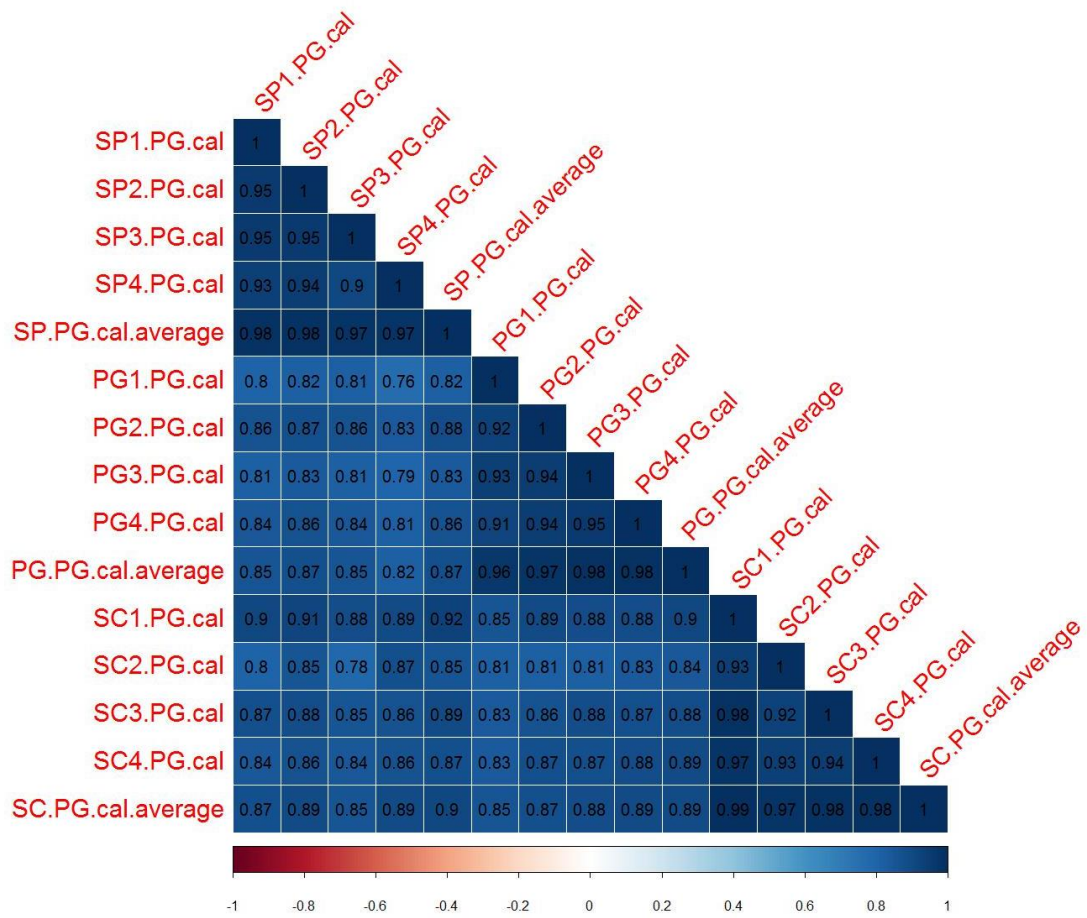


Figure S2: Cattle: Correlations among RTL measurements calculated using a Puregene extracted calibrator

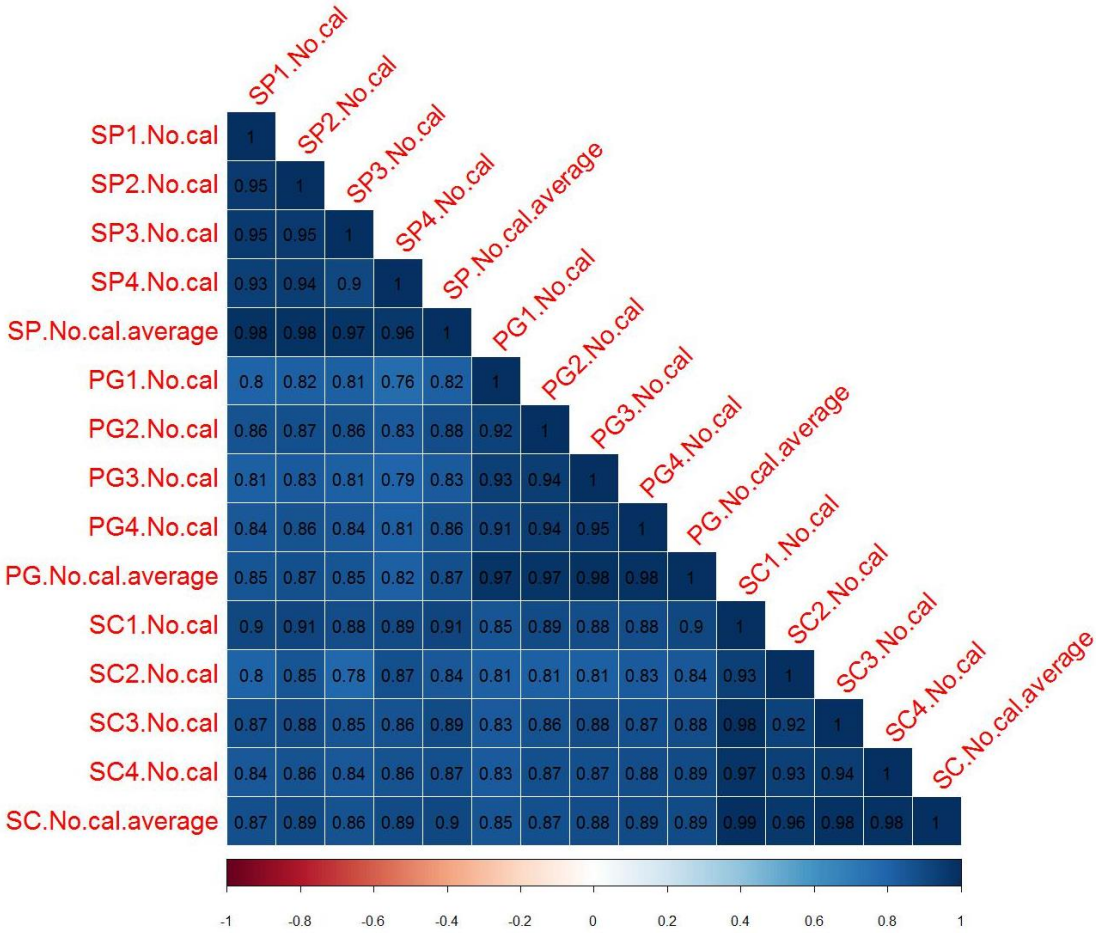


Figure S3: Cattle: Correlations among RTL measurements derived without a calibrator

2.16.2. Sheep

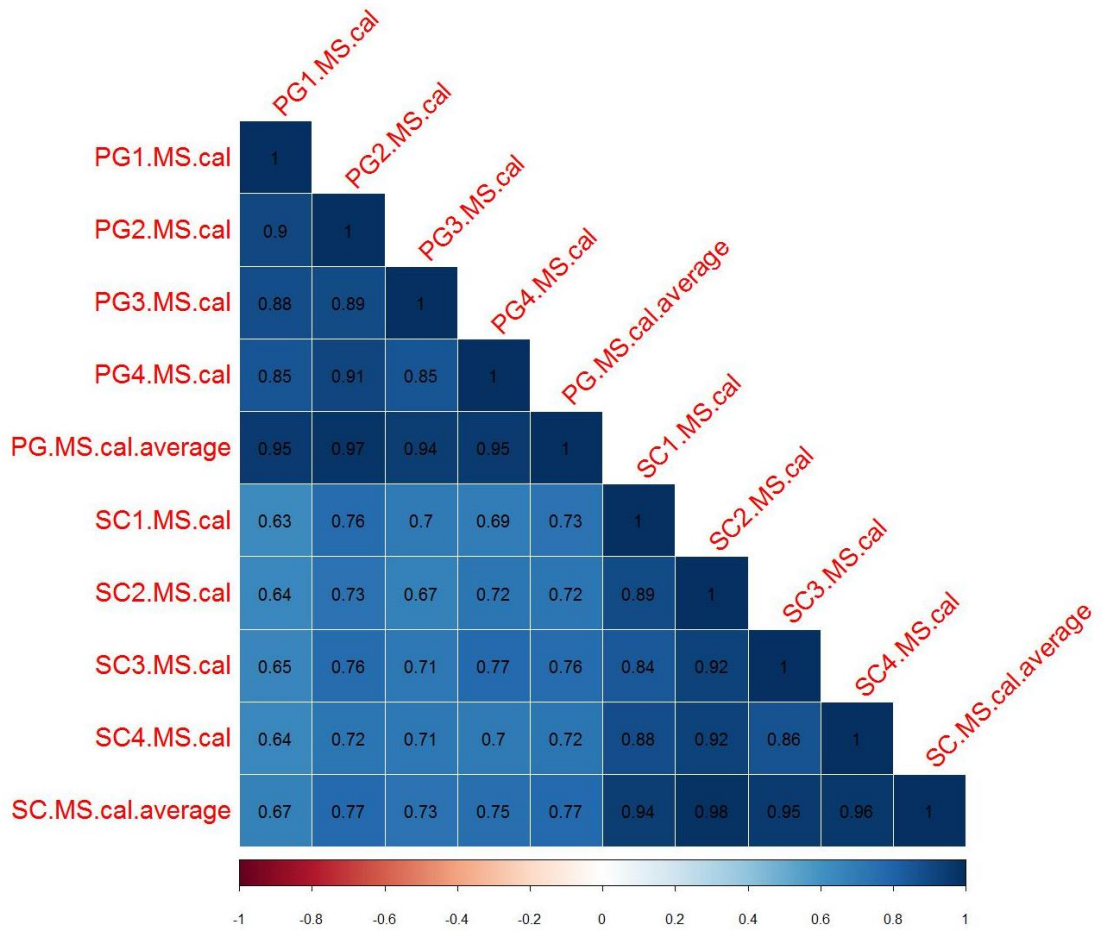


Figure S4: Sheep: Correlations among RTL measurements calculated using a method-specific calibrator

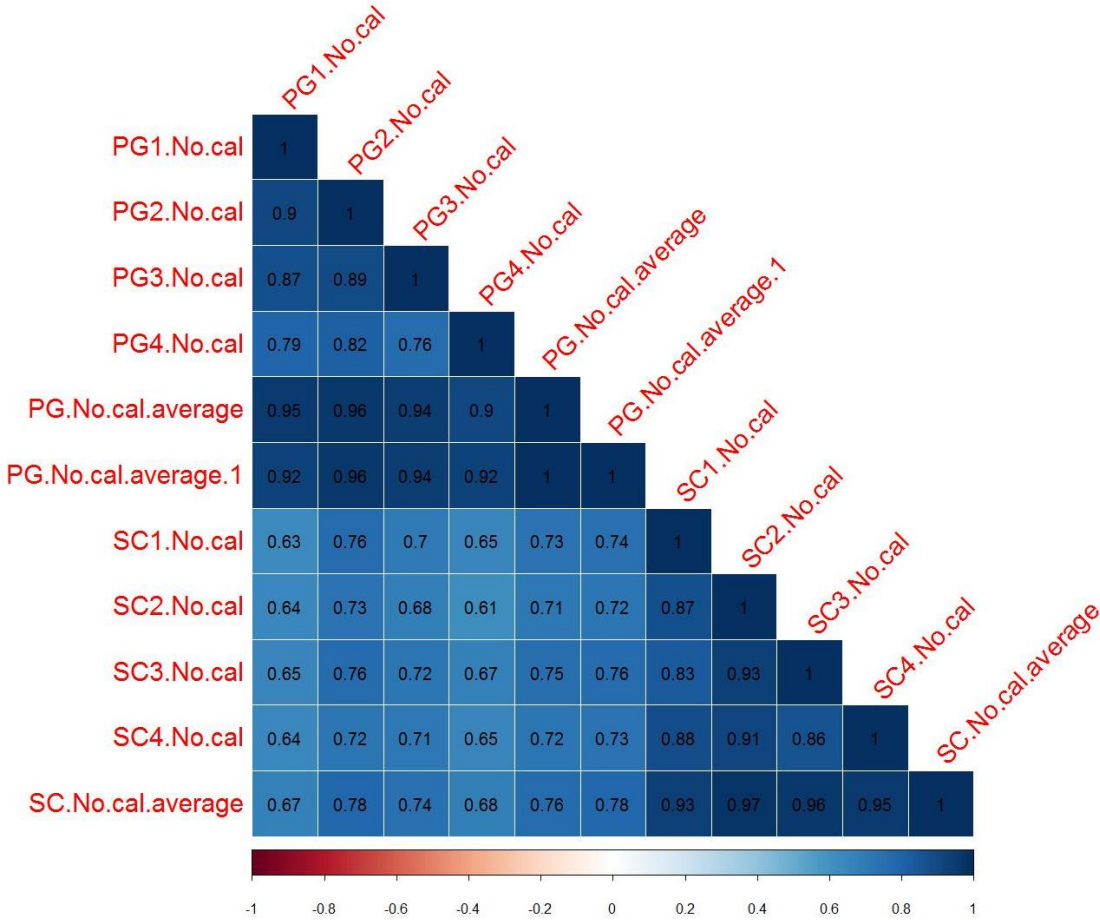


Figure S5: Sheep: Correlations among RTL measurements derived without a calibrator

Chapter 3

Bovine telomere dynamics and the association between telomere length and productive lifespan

3.1. Prefix

The previous chapter presented robust methods for the measurement of RLTL in dairy cattle. These methods were used for the large-scale telomere project presented in the current chapter to better understand RLTL dynamics with age, factors that influence RLTL and the association between RLTL and productive lifespan. Also, the first heritability estimates of RLTL in a livestock species are presented.

This chapter has been submitted as

Seeker, L. A., Ilska, J. J., Psifidi, A., Wilbourn, R.V., Underwood, S., Fairlie, J., Holland, R., Froy, H., Salvo-Chirnside, E., Bagnall, A., Whitelaw, B., Coffey, M., Nussey, D. H., Banos, G. (2017). Bovine telomere dynamics and the association between telomere length and productive lifespan.

for publication to the journal Scientific Reports where it is currently under peer review.

I conducted the experimental and analytical work under guidance from the supervisors and wrote the manuscript with the help of supervisors and co-authors.

Supplementary files 1 of the submitted paper can be found in chapter appendix 1 (3.11). To comply with journal guidelines, the results and discussion of this chapter are presented before the materials and methods.

3.2. Abstract

Average telomere length (TL) in blood cells has been shown to decline with age in a range of vertebrate species, and there is evidence that TL is a heritable trait associated with late-life health and mortality in humans. In non-human mammals, few studies to date have examined life-long telomere dynamics and no study has estimated the heritability of TL, despite these being important steps towards assessing the potential of TL as a useful biomarker of productive lifespan and health in livestock species. Here we measured relative leukocyte telomere length (RLTL) in 1,328 samples from 308 Holstein Friesian dairy cows and in 284 samples of 38 female calves. We found that RLTL declines after birth but remains relatively stable in adult life. We also calculated the first heritability estimates of RLTL in a livestock species which were 0.32 (SE=0.08) and 0.38 (SE=0.03) for the cow and the calf dataset, respectively. RLTL measured at the ages of one and five years, but not at other ages, were positively correlated with productive lifespan ($p < 0.05$). We conclude that bovine RLTL is a heritable trait, and its association with productive lifespan may be used in breeding programmes.

3.3. Introduction

Telomeres are structures at the ends of linear chromosomes that consist of repetitive DNA nucleotides and attached proteins of the shelterin complex (Blackburn & Gall 1978; De Lange 2005). They are crucial for chromosomal integrity and pairing of homologous chromosomes during meiosis (Blackburn 1991; Zakian 1989). In cultured cells telomeres shorten with every cell division (Harley, Futcher & CW. Greider 1990) due to the end replication problem (Watson 1972; Olovnikov 1973). When telomeres become critically short and repair mechanisms are not activated, the cell enters a state called replicative senescence where it is unable to divide further (Campisi 1997). Telomere shortening is a hallmark of cellular ageing and also seems to be associated with organismal ageing (López-Otín et al. 2013) with most young individuals having longer telomeres than old individuals of the same species (Barrett et al. 2013; Beirne et al. 2014; Aviv et al. 2009; Salomons et al. 2009). However, TL dynamics across different ages vary among species. In humans, telomere attrition with age is usually described to follow a general pattern with three stages: 1) fast telomere attrition in early life, 2) slower attrition or plateau in young adulthood and middle age, and 3) rapid depletion at older ages (Aubert & Lansdorp 2008; Frenck et al. 1998). Rapid telomere attrition during early life has

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been observed in a wide range of species, including Soay sheep (Fairlie et al. 2015), baboons (Baerlocher et al. 2007), European shags (Hall et al. 2004) and wandering albatrosses (Hall et al. 2004). However, the pattern of change in TL with age during adult life seems to vary. Adélie penguins, common terns, tree swallows, zebra finches and great frigatebirds show continuing telomere attrition at adult age (Hausmann et al. 2003; Juola et al. 2006), whilst TL remains stable in adult European shags and wandering albatrosses (Hall et al. 2004), and it actually increases in adult edible dormice (Hoelzl et al. 2016).

Average TL, measured in leukocytes in mammals and erythrocytes in non-mammalian vertebrates, has emerged as a potentially important biomarker of health and ageing across disciplines including epidemiology, biomedicine, ecology and animal welfare (Bateson 2016; Monaghan et al. 2018; Monaghan & Ozanne 2018; Olsson et al. 2018; Aviv & Shay 2018; Risques & Promislow 2018; Harrington & Pucci 2018; Young 2018). There is mounting evidence from across a range of species that blood cell TL is both heritable and predictive of subsequent health and mortality risk (Dugdale et al. 2017; Boonekamp et al. 2013; Wilbourn et al. 2018). Heritability estimates of TL have been calculated using parent-offspring regressions, correlations between twins or pedigree-based 'animal' models for a variety of species including humans, sand lizards and a range of bird species. Most studies suggest that variance in TL is under some degree of genetic control (Dugdale et al. 2017).

Short telomeres have been shown to be associated with higher mortality in species such as zebra finches (Heidinger et al. 2012), semi-feral Soay sheep (Fairlie et al. 2015) and humans (Bakaysa et al. 2007; Boonekamp et al. 2013). However, other studies found no relationship between TL and lifespan (Hovatta et al. 2012; Raymond et al. 2012). A meta-analysis of human studies found evidence that the association between TL and mortality risk was only present at younger adult ages (Boonekamp et al. 2013), whilst a meta-analysis of non-human vertebrate studies found a significant overall association between TL and survival despite considerable variation among studies (Wilbourn et al. 2018).

Interest in the potential application of TL as a biomarker of health and welfare within the livestock industry is growing (Bateson 2016; Brown et al. 2012). In particular, the improvement of so-called 'functional longevity' (which is productive lifespan corrected for milk yield) is currently a chief breeding goal in dairy cattle, because it

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would reduce the requirement for replacement heifers, minimise waste, improve animal welfare, and decrease greenhouse gas emissions and farming costs (Garnsworthy 2004). Improving functional longevity is difficult to achieve with conventional selective breeding for several reasons: phenotypes are recorded at the end of life which causes long generation intervals and slows genetic progress (Visscher et al. 1999); the recording of specific phenotypes such as reasons for culling is laborious and often not practical on a busy commercial farm; functional longevity in cattle is known to have a low heritability of approximately 0.01-0.06 (Pritchard et al. 2013). Therefore, an early life biomarker that is heritable and correlated with functional longevity would benefit the dairy industry tremendously, as it would enable the selection of animals in early life based on the biomarker measurement, long before they express the phenotype of interest (productive lifespan).

Currently, very little is known about TL dynamics across different ages in cattle. In two cross-sectional studies, young cattle were found to have longer telomeres than older animals suggesting that bovine telomeres might shorten with age (Brown et al. 2012; Miyashita et al. 2002). Based on those studies it can be hypothesised that cattle would follow a human-like TL dynamics with age where telomeres shorten quickly after birth and at a slower pace thereafter.

As mentioned, for TL to be a useful biomarker for selective breeding purposes, it must be heritable. Heritability estimates for TL in non-human mammals outside the laboratory are so far missing from the literature. However, based on detection of low to moderate heritability of TL in humans and wild bird studies and the fact that domestic cattle live in a more controlled environment than either of the former species, we hypothesise that RLTL should be at least moderately heritable in cattle as well.

Crucially, the relationship between RLTL at different life stages and productive or functional longevity in dairy cattle has not been explored. Obviously, productive lifespan of dairy cows differs from longevity measurements in humans and wild animals because most cows are culled by humans. However, cows are not randomly chosen for culling, which is usually based on poor fertility or health. The only study that has addressed the association of TL with productivity in dairy cattle so far indeed found suggestive evidence that animals with short telomeres were

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more likely to be culled within one year of measurement (Brown et al. 2012). However, the hypothesis that TL measured during early life in dairy cattle is predictive of future productive longevity remains to be tested.

In the present study, we repeatedly measured individual RLTL at different life stages in a large, well-monitored and pedigreed population of Holstein Friesian dairy cattle (Veerkamp et al. 1994). Animals were kept at the Crichton Royal research farm in Dumfries (Scotland). They represented two genetic lines (selected for high milk fat and protein yield (S) vs. control (C)) that were randomly allocated to two different diets (High forage (HF) vs. low forage (LF)) which allowed us to investigate the effect of genetic and nutritional factors on RLTL.

Our objectives were to test the following hypotheses (i) RLTL shortens with age in dairy cattle and telomere attrition is fastest shortly after birth, (ii) factors other than age such as season of birth, genetic background and diet also affect RLTL, (iii) RLTL is a heritable trait in dairy cattle and (iv) RLTL in early life is predictive of productive lifespan.

3.4. Results

Table 1 shows the number of RLTL measurements per animal available in the cow and the calf dataset, while Tables S1 and S2 summarise other relevant information.

Table 1: Characterisation of the cow and the calf datasets, showing the number of animals and the number of RLTL measurements per animal (1-9). All animals had an early life sample taken within two weeks of birth. Subsequent samples were taken approximately yearly and monthly for the cow and the calf datasets, respectively.

Dataset	Total number of animals	Total number of RLTL measurements	Number of RLTL measurements per animal								
			1	2	3	4	5	6	7	8	9
Cow dataset	308	1,328	3	28	38	106	83	35	14	1	0
Calf dataset	38	284	0	0	1	2	3	5	4	7	16

3.4.1. RLTL changes with age at the population level

We observed a considerable decline in RLTL within the first year of life (estimate=-0.11, SE=0.007, $p < 0.001$, Cohen's $D = -0.44$) with the most rapid telomere depletion shortly after birth (Fig. 1). There was little visual evidence of a systematic change in RLTL with increasing age in animals older than one year (Fig.1), which is consistent with the finding that modelling age as a two level factor (younger vs. older than one month of age) fitted the cow dataset best (based on AIC; Table 2). Within age group, RLTL was highly variable (CV= 0.16 and 0.18 for the cow and calf datasets, respectively). In fact, the distribution of measurements of neonates in the cow dataset overlapped that of the oldest animals (Fig. 1).

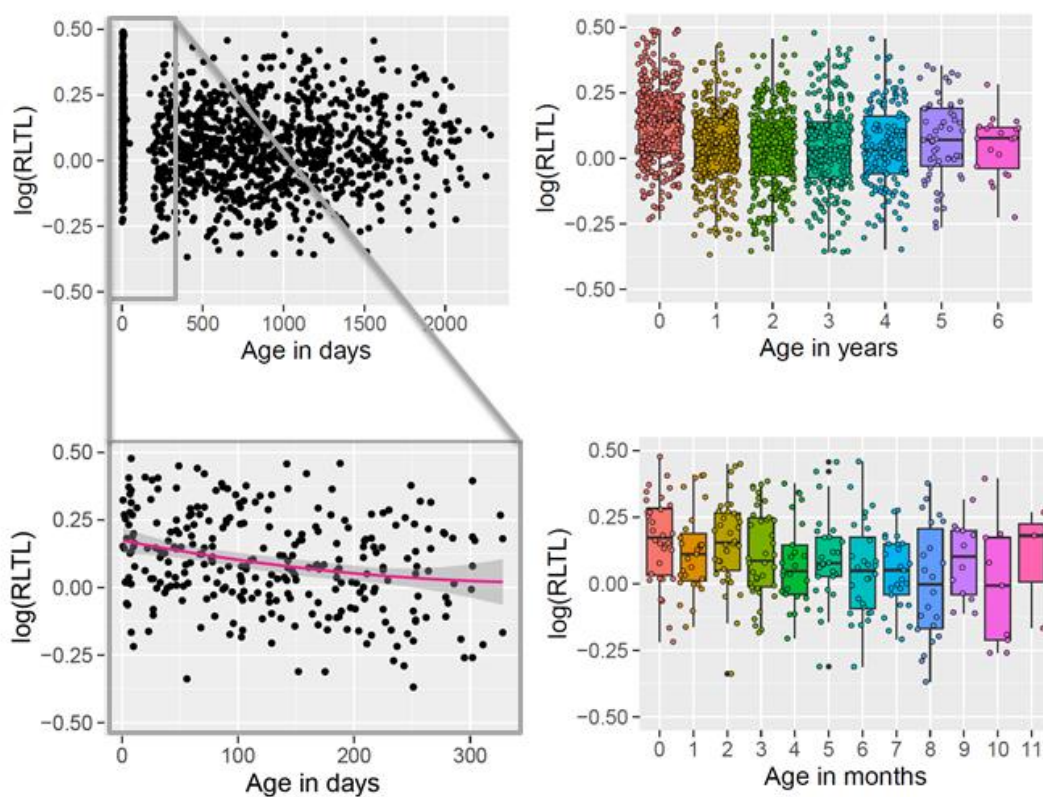


Figure 1: Impact of age on relative leukocyte telomere length (RLTL). Top row: Cow dataset. Log transformed RLTL over age in days (left panel) and years (right panel). Bottom row: Calf data set. Log transformed RLTL over age in days (left panel) and months (right panel). In the bottom left panel a quadratic function of age is visualised.

The cow and the calf datasets complement each other nicely in the present study. While the cow dataset provides information about RLTL throughout productive life, it

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does not include measurements between two and six months of age. Therefore, although we see a difference in average RLTL between neonates and older animals, we cannot be certain about the dynamics of the decline within the first few months of life. This gap is filled by the calf dataset which provides repeated measurements in early life and shows that RLTL declines with age at a slightly faster rate shortly after birth than closer to the age of one year. In the calf dataset this dynamic is best captured by a quadratic function of age or by modelling age as a two level factor (younger vs. older than four months) (Table 2), Within two AIC units the simpler model is preferred over the more complicated one (Burnham & Anderson 2002; Arnold 2010; Froy et al. 2015). We decided to include a quadratic function of age in the final model for the analysis of the calf dataset.

Table 2: Comparison of models with different functions of age. Delta AIC values are expressed in relation to the best fitting model (age as a 2-level factor and quadratic in cow and calf datasets, respectively). A delta AIC of at least 2 units corresponds to a significant difference between models ($p < 0.05$). Within two units the simpler model is preferred over the more complicated one.

Function of age	AIC	Delta AIC
Cow dataset		
/	-1431.4	193.7
linear	-1508.2	116.9
quadratic	-1575.4	49.7
cubic	-1598.6	26.5
quartic	-1605.0	20.1
age as 2-level factor	-1625.1	0
Calf dataset		
/	-255.89	20.74
Linear	-273.7	2.93
Quadratic	-276.63	0
Cubic	-277.64	-1.01
Quartic	-275.85	0.78
Age as 2-level factor	-276.92	-0.29

3.4.2. Factors affecting RLTL independently of age

The final model for the analysis of the cow dataset contained the animal identity as a random effect and the following significant fixed effects: age as a two-level factor (younger vs. older than one month; estimate difference = -0.11, SE = 0.007, $p < 0.001$), birth year of cow ($p = 0.004$), qPCR plate ($p < 0.001$) and qPCR row ($p < 0.001$) (Supplementary Table S5). For the analysis of the calf dataset, the final model

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included the animal identity as a random effect, a quadratic function of age (linear estimate=-0.606, SE= 0.138, $p<0.001$ and quadratic estimate= 0.294, SE=0.13, $p=0.031$), birth weight ($p=0.04$) and birth season ($p= 0.04$) of the calf, qPCR plate ($p<0.001$), and qPCR row ($p<0.01$) as fixed effects. Heavier calves had marginally shorter RLTL at birth (estimate difference=-0.005, SE= 0.002, $p=0.045$) and calves born over the winter months (October to March) had longer telomeres (estimate difference=0.069, SE= 0.033, $p=0.041$). Supplementary tables S5 and S6 and supplementary figures S1- S4 summarise all significant effects.

Interestingly, neither in the cow dataset nor in the calf dataset was the genetic group of the animal significantly associated with RLTL. Also, there was no statistically significant relationship between the feed group and RLTL in the cow dataset.

3.4.3. Estimation of genetic and environmental variance components

The animal effect in the model included pedigree information, which enabled the estimation of the genetic variance for RLTL. A permanent environment random effect was also fitted to account for common non-genetic variance of repeated measures within individual animals. Estimates of variance components and genetic parameters are shown in Table 3. The variance due to permanent environment was practically zero. Therefore, the permanent environment effect on RLTL in our datasets is negligible. Heritability estimates of RLTL were 0.38 (SE= 0.03) and 0.32 (SE= 0.08) for the cow and the calf datasets, respectively.

Table 3: Variance components and genetic parameters. Estimates are followed by standard errors in brackets.

dataset	phenotypic variance	genetic variance	permanent environment variance	residual variance	permanent environment	repeatability	heritability
cow	0.0037 (1.9 E-04)	0.0014 (0.0002)	1.8 E-10 (8.1 E-12)	0.0023 (1.03 E-04)	0.0 (0.0)	0.3832 (0.03)	0.3832 (0.03)
calf	0.0047 (5.6 E-04)	0.0015 (0.0005)	2.5 E-9 (2.3 E-10)	0.0032 (2.97 E-04)	0.0 (0.0)	0.3231 (0.08)	0.3231 (0.08)

1.1.1. Relationship between RLTL and productive lifespan

Livestock animals are usually culled at the end of their life. Therefore, their longevity measures have to be defined differently than for members of natural populations. We define “productive lifespan” as the survival time from birth to culling measured in days. “Functional longevity” is defined as productive lifespan corrected for milk yield and is a measure that is often used in genetic studies of dairy cattle. A total of 244 out of 308 animals in the cow dataset were culled thereby providing phenotypes for productive lifespan measurements. For most of them (220 out of 244) a precise culling reason was recorded (Supplementary Table S7). The majority of animals were culled involuntarily, particularly due to fertility problems, mastitis or lameness. Productive lifespan ranged from 17 to 2,823 days (mean = 1,477 days) (Figure S6), which means that on average cows were culled during their third lactation. There was no difference in productive lifespan between the two genetic lines (Welch two sample t-test: $t = -0.8954$, $df = 242$, $p\text{-value} = 0.3714$) and between the two feed groups (Welch two sample t-test: $t = -1.1235$, $df = 170$, $p\text{-value} = 0.2628$).

In the present study we were interested in the potential use of RLTL as an early life trait associated with productive lifespan. Therefore, the subsequent analyses were focused on RLTL measurements at birth and at the age of one year. We defined functional longevity as productive lifespan adjusted for milk yield. For this reason, we fitted a linear model with productive lifespan as the response variable and the average milk yield over the first four lactations as a covariate. Subsequently, we fitted the animal’s RLTL measurement at birth or at the age of one year (separate analyses) as a second covariate to the model to assess its relationship with functional longevity. In either case, RLTL had been first pre-adjusted for qPCR plate and row by fitting those factors as fixed effects and using the residuals of the model for further analysis. Because animals were required to have both productive lifespan and milk yield records over the first four lactations available, these analyses were based on 143 and 136 animals with RLTL measurements at birth and one year of age, respectively. Milk yield significantly affected lifespan of those animals (estimate= 81.13, SE=13.92, $p < 0.001$) but RLTL neither at birth (estimate=-11.10, SE= 329.77, $p=0.973$) nor at the age of one year (estimate= 355.91, SE= 394.42, $p=0.368$) had a significant effect.

However, the analysis of functional longevity excluded animals that might be of particular interest: animals that died at a young age were excluded for not having

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productivity records; also animals that were still alive and belonged to the group of animals with the best productive lifespan were excluded, because they did not have a productive lifespan measurement yet. Therefore, we tested the phenotypic correlation between productive lifespan and functional longevity ($r=0.9$, $p<0.001$, see Supplementary Fig. S5) and found that productive lifespan may be used as a proxy trait for functional longevity in the present study. This allowed us to include all animals that died before generating productivity measurements. Also, we considered a Cox proportional hazard analysis that enabled us to include all animals that were still alive by including a censoring group in the analysis. With those measures we increased our sample size from 143 to 305 RLTL measurements at birth and from 136 to 284 measurements at the age of one year. With the Cox proportional hazard analyses we investigated the survival time in years after sampling. We found that RLTL at birth was not a predictor of survival ($p=0.234$), but there was a significant positive linear relationship between RLTL at the age of one year and subsequent survival (estimate = -1.974, SE= 0.708, $p=0.005$). The estimate is negative, because it describes the relation between RLTL and mortality which is the opposite of survival. RLTL at subsequent ages were also tested. RLTL at the ages of 2-4 years did not relate to productive lifespan, but RLTL at the age of 5 years was positively correlated with subsequent survival ($N=53$, estimate=-3.267, SE= 1.346 , $p=0.015$) (Table 4).

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Table 3: Relationship of RLTL measured at different ages with subsequent survival. CI: Confidence interval; *significant at $\alpha=0.05$ level, **significant at $\alpha=0.01$ level.

RTL at age in years	N	Beta coefficient (SE)	Hazard Ratio (95% CI)	Wald statistic	P-value	
0	305	0.768 (0.661)	2.155 (0.591-7.866)	1.35	0.245	
1	284	-1.974 (0.708)	0.139 (0.035-0.556)	7.78	0.005	**
2	261	0.763 (0.682)	2.144 (0.563-8.16)	1.25	0.264	
3	220	-1.285 (0.744)	0.277 (0.064-1.188)	2.99	0.084	
4	208	-1.147 (0.763)	0.318 (0.071-1.416)	2.26	0.133	
5	53	-3.267 (1.346)	0.038 (0.003-0.533)	5.89	0.015	*

The relationship between RLTL at the age of one year (grouped into tertiles) and productive lifespan is visualised in Fig. 2.

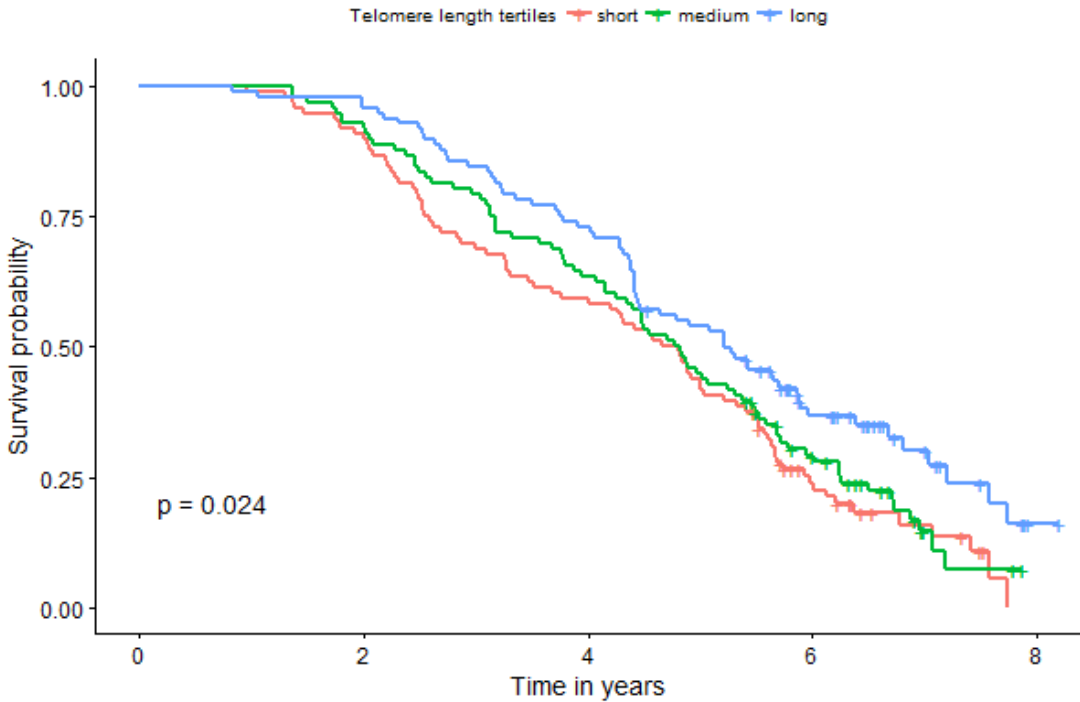


Figure 2: Kaplan-Meier plot for survival probability in relation to telomere length tertile at the age of 1 year.

3.5. Discussion

This is the first large-scale longitudinal study of RLTL dynamics conducted in farm animals and one of the few lifelong studies reported in any species. The combination of calf and cow datasets provided valuable information allowing us to better understand telomere dynamics in early life and throughout the animal's productive life, respectively. Our results show that average RLTL declines within the first year of life but remains relatively stable thereafter. Telomere attrition shortly after birth has been reported in other mammals such as humans (Aubert & Lansdorp 2008; Frenck et al. 1998; Rufer et al. 1999), baboons (Baerlocher et al. 2007) and Soay sheep (Fairlie et al. 2015) as well as some birds such as Seychelles warblers (Barrett et al. 2013), European starlings (Nettle et al. 2017), corvid (Salomons et al. 2009) and Jackdaws (Boonekamp et al. 2014), and is probably due to the high number of cell divisions that are necessary for the body to grow quickly during that time. Also, postnatal maturation of the immune system and the sudden challenge to fight off pathogens after birth might cause quick telomere depletion within the first months. Varying results have been reported about telomere dynamics in adult life in different species. While some studies in humans, badgers and birds show a linear telomere decline throughout adult life (Aubert & Lansdorp 2008; Beirne et al. 2014; Barrett et al. 2013; Rufer et al. 1999; Salomons et al. 2009), a study on the semi-feral Soay sheep reported more complex dynamics: telomeres shortened within the first four months of life, then lengthened until the age of 5 years and shortened afterwards again (Fairlie et al. 2015). Other studies in mammals and birds report similar results to ours with no relation between age and TL after the initial decline early in life (Hall et al. 2004; Baerlocher et al. 2007; Pauliny et al. 2006). A particularly interesting telomere changing pattern has been observed in edible dormice: after the initial decline in early age, telomeres elongate considerably in adult life (Hoelzl et al. 2016). Those are a few examples that illustrate that telomere dynamics across life stages vary among species. Our results do not support former studies on TL in cattle that reported an age-dependent decline in adult life (Miyashita et al. 2002; Brown et al. 2012). Former studies may have found TL attrition with age, because they included older animals in their analyses (maximum age of 13 or 14 years (Miyashita et al. 2002; Brown et al. 2012) compared to six years in the present study). It is possible that bovine RLTL dynamics are similar to those observed in humans with fast telomere attrition in early life, a plateau in

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younger adulthood and possibly a second decline later in life that we failed to detect in the present study because we did not test animals that were older than six years.

To our knowledge we report in the present study the first heritability estimates for RLTL in a livestock species. Presence of significant genetic variation and heritability of bovine RLTL suggests that the trait may be altered with selective breeding; heritability estimates of RLTL in cattle were relatively high (0.31-0.39) and within the range of estimates calculated using linear mixed models in other species such as humans ($h^2=0.28-0.76$) (Faul et al. 2016; Njajou et al. 2007; Bakaysa et al. 2007; Zhu et al. 2013; Blackburn et al. 2015; Hjelmborg et al. 2015; Lee et al. 2013; Honig et al. 2015) and birds ($h^2=0-0.999$) (Asghar et al. 2015; Voillemot et al. 2012; S Reichert et al. 2014; Atema et al. 2015; Becker et al. 2015). The human studies benefitted in comparison to the bird studies from larger sample sizes (Dugdale et al. 2017).

One factor that might affect our heritability estimates of RLTL is the potential presence of interstitial telomeres in the cattle genome. Interstitial telomeres are telomeric sequences that are not located at the ends of chromosomes but between centromeres and telomeres (Lin & Yan 2008; Mohan et al. 2011; Bolzán & Bianchi 2006; Foote et al. 2013; Matsubara et al. 2015; Ruiz-Herrera et al. 2002). Interstitial telomeres are believed to be constant in their amount between tissues and also to remain constant in their length with ageing. Therefore, their presence could lead to an over-estimation of the heritability of RLTL. However, little is known about the presence of interstitial telomeres in the cattle genome to date. For future studies it would be interesting to measure bovine TL using a method that exclusively measures telomeric sequences at the ends of chromosomes (in gel TRF (Hausmann & Mauck 2008)) which will provide more accurate heritability estimates if interstitial telomere are present in the cattle genome and if their amount varies between individuals.

The two genetic groups in our study population, which had been selected to differ considerably in milk production, did not differ in their mean RLTL. Based on these results an unfavourable genetic correlation between TL and productivity is highly unlikely which is desirable because it implies that TL may be altered with genetic selection within a breeding programme without reducing the milk yield of cows.

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We found that feed group of the animals did neither affect RLTL nor the productive lifespan. It has been shown that calorie restriction improves longevity in many species (Bordone & Guarente 2005) and also that diet affects TL in humans (Cassidy et al. 2010; Mirabello et al. 2009; Nettleton et al. 2008). However, the human diet varies a lot more between individuals and there is a greater potential for truly unhealthy and harmful eating habits in humans than in cattle. For example, Nettleton et al. (2008) found a negative effect of the consumption of processed meat on TL, while Cassidy et al. (2010) found a positive effect of dietary fibre intake (Nettleton et al. 2008; Cassidy et al. 2010). While the two feed groups in the present study differ in energy content as defined in separate feed experiments on this population (Pollott & Coffey 2008; Bell et al. 2010; Ross et al. 2014), they can both be considered as healthy and fibre rich.

In the analysis of the calf dataset we found a significant relationship between birth weight and RLTL, with heavier calves having shorter telomeres. This is in accordance with the theory that faster body growth requires more cell divisions and, therefore, bigger sized individuals of the same species have shorter telomeres (Stindl 2004). However, it is in contrast to former studies in humans that found no relation of birth weight and TL (Okuda et al. 2002; Akkad et al. 2006) or longer telomeres in bigger and heavier babies (de Zegher et al. 2016). In our study, birth weight might be confounded with gestation length. Heavier calves may have been born past their due date and their having shorter telomeres could be a reflection of fast age-dependent telomere depletion before birth which usually happens during the first days of life.

It is not clear if the birth year effect on RLTL in the cow dataset reflects random noise associated with measurement error or true biology. Year-dependent changes in weather, food quality or other environmental factors may affect RLTL measurements systematically. Birth cohort effects on RLTL have been observed before in wild Soay sheep (Fairlie et al. 2015) which could support the presence of a true biological effect of birth year on the animals' RLTL. However, in contrast to Soay sheep, cattle in our study were born and lived in a controlled environment which makes biological cohort effects less likely. The observation of a potential season effect on RLTL of calves has not been reported before and might be explained by different pregnancy conditions either due to more daylight over the summer months or due to more fresh feed available during the grazing period.

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Stress (Entringer et al. 2013; Entringer et al. 2011) and nutrition (Tarry-Adkins et al. 2008) during pregnancy have been reported to affect TL in humans and rats respectively. Also, experimentally elevated stress hormones during pregnancy and early post-natal life conditions have been associated with shorter telomeres or faster telomere shortening in birds (Herborn et al. 2014; Tissier et al. 2014; Boonekamp et al. 2014; Voillemot et al. 2012). Besides those studies, little is known about factors during pregnancy and early life that affect the offspring's TL and more specifically designed studies are required to gain a better understanding. For future studies on RLTL it would be interesting to investigate the possible effect of the dam's feed group on the calves' RLTL, which may be associated with the birth season effect. Also, the inclusion of a possible maternal effect in future analyses would be interesting.

No significant association of RLTL either at birth or at one year of age with functional longevity (productive lifespan adjusted for milk yield) was found in the present study. The sample size might have been too small to detect such an effect. However, when we included more animals by investigating productive lifespan as a proxy for functional longevity and by introducing a censoring group in the Cox proportional hazard analysis to include animals that were still alive, we found that animals with the longest telomeres at the ages of one and five years lived significantly longer ($p < 0.05$). There was no significant signal for RLTL at birth and other ages. Former studies on the potential value of telomeres as a biomarker for lifespan in humans and laboratory animals have shown mixed results: whilst some studies found a relationship between short telomeres and reduced lifespan or the early onset of age related symptoms and diseases (van der Harst et al. 2007; Hochstrasser et al. 2011; Willeit et al. 2011) other studies could not find a relationship (Hovatta et al. 2012; Raymond et al. 2012; Martin-Ruiz et al. 2005; Bischoff et al. 2006). Two meta-analyses on humans and non-human vertebrates suggest an overall significant positive relationship between TL and lifespan (Boonekamp et al. 2013; Wilbourn et al. 2018). In dairy cattle, the only other study on RLTL in relation with productive lifespan so far showed a significant but weak association (Brown et al. 2012). Cows with short telomeres were more likely to be culled within the next year regardless of their age at sampling. It is not clear why we detected an association between TL and productive lifespan at the ages of one and five years but not at other ages.

In order to clarify if RLTL could be used as a biomarker for breeding for improved productive lifespan it would be interesting to investigate the genetic correlation between RLTL and productive lifespan. Genetic correlations between two traits estimated with bivariate analyses, in contrast to phenotypic correlations estimated in the present study, will not be affected by environment effects and might therefore deliver different results.

There is growing interest in the change in TL rather than single measurements at specific ages in association with lifespan. Several studies in birds have indeed shown that the rate of change in TL might be more strongly associated with lifespan than mean TL measurements (Boonekamp et al. 2014; Barrett et al. 2013; Salomons et al. 2009; Nettle et al. 2017). This type of association in dairy cattle warrants further investigation.

3.6. Materials and methods

3.6.1. Study system & sample selection

Holstein Friesian dairy cattle used in the present study belong to the herd kept at the Crichton Royal research farm in Dumfries (Scotland) where they are closely monitored and repeatedly sampled for a broad range of scientific studies, including feeding, greenhouse gas emission and genetics experiments. Since 1976 two distinct genetic lines are maintained at the farm: a selection (S) line and a control (C) line (Veerkamp et al. 1994). Cows of the S line are amongst the highest yielding dairy cows in the UK, due to a careful selection of breeding bulls with the highest genetic merit for total milk, fat and protein production internationally. Cows of the C line have are deliberately kept at an average UK performance level. Animals of the two genetic lines are randomly allocated to two different feeding groups after first calving (calf birth): a high forage (HF) and a low forage (LF) diet group. While the HF group is turned out to the fields during the summer months, the LF group is housed continuously. The LF diet is based on by-products of plants farmed for human consumption and is richer in energy and protein content than the HF diet which is based on farm grown fibre-rich feed. Feeding affects the metabolism and milk yield of a dairy cow: a cow of the S line has a targeted milk yield of 7,500 litres per year when managed on a HF diet and of 13,000 litres per year when managed on a LF diet. The farm keeps 50 milking cows of each genetic and feeding group at all times

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which makes it ca. 200 milking animals in total. The monitoring of the animals includes regular measurement of milk yield, body condition score, body weight, health events, feed intake, fertility measurements, calving records, productive lifespan measures, reasons for culling etc. Cows stay in the experiment for three lactations or five years. Afterwards, they are transferred to the Acrehead Dairy Unit where they are still used commercially. While the feeding experiment stops at that point and animals are monitored less intensively, regular blood samples and basic records are still collected until culling.

3.6.2. Blood sampling & sample selection

A blood sample archive for the cattle of the Crichton herd was started in 2004 and routine sampling protocols include initial blood sampling within the first two weeks of life and an annual sampling of all animals in spring. When possible, an additional sample is taken close to the animal's planned culling (removal from the herd). Whole blood samples are taken by venepuncture (V. jugularis for calves and V. coccygealis for adults) and EDTA is used as an anticoagulant. Samples are stored at -30°C. In the present study we used these blood samples to extract DNA and measure TL of individual animals. In the first instance, we selected 1,336 blood samples from 308 individual cows taken at different ages (cow dataset). Since few samples were collected on these animals within the first year of life, we collected an additional 284 blood samples from 38 female calves over the course of their first year of life, at approximately monthly intervals (calf dataset).

3.6.3. Ethics statement

Blood sampling was approved by the SRUC Animal Experimentation Committee and conducted in accordance with UK Home Office regulations (UK Home Office Project License Number: PPL 60/4278 Dairy Systems, Environment and Nutrition).

3.6.4. DNA extraction

Leukocyte DNA was extracted using spin columns of the DNeasy Blood & Tissue kit (QIAGEN). We followed the manufacturer's protocol for the extraction from whole blood samples with certain alterations (Seeker et al. 2016). DNA yield and purity were measured for each sample on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) with the software NanoDrop 2000 and the DNA integrity of each sample was tested with gel electrophoresis (as recommended by Kimura et al., 2010). We processed samples which met the following requirements: yield > 20

ng/ μ l, 260/280 ratio > 1.7 and 260/230 ratio >1.8, integrity gel score <3 (according to Seeker et al., 2016). We extracted DNA with acceptable quality from 1,612 out of 1,620 whole blood samples (1,328 samples belonged to the cow dataset and 284 samples to the calf dataset).

3.6.5. Quantitative polymerase chain reaction (qPCR)

Average RLTL was measured by qPCR (Cawthon 2002) as described in Seeker et al. (2016). Average RLTL is expressed as the amount of telomeric DNA in relation to the amount of a reference gene that is constant in copy number. Both the telomere reaction and the reference gene reaction were performed in different wells but on the same qPCR plate (monoplex qPCR). One sample –the so called calibrator or golden sample- was repeated on each plate and included in the calculation of RLTL to correct for random measurement error and to allow a comparison of RLTL measurements from different qPCR plates. The calibrator sample was extracted using the same protocol as for the samples in the study (QIAGEN Blood & Tissue kit spin columns) (Seeker et al. 2016).

The master mixes for the telomere and the reference gene reaction contained 5 μ l of LightCycler 480 SYBR Green I Master (Roche) per well. For the telomere amplification, 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') (Epel et al. 2004) primers were used at a concentration of 900 nmol. They were manufactured and purified with high performance liquid chromatography by Integrated DNA Technologies (IDT, Glasgow, UK). For the reference gene amplification beta-2-microglobulin (B2M) primers (Primerdesign, accession code NM_001009284) were used at a concentration of 300 nmol (Seeker et al. 2016). Nuclease free water was added to the master mixes to allow for a final volume of 10 μ l per qPCR plate well. The reference gene B2M has been previously tested against other candidate genes for a consistent copy number using the qbase+ software by Primerdesign and showed stable qPCR results (Seeker et al. 2016). This gene has been used for RLTL measurement in different ruminant species such as Soay sheep (Fairlie et al. 2015; Froy et al. 2017; Watson et al. 2017), roe deer (Wilbourn et al. 2017) and Holstein Friesian dairy cattle (Seeker et al. 2016).

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Samples were randomly allocated to a qPCR plate and well. A 96 well sample plate was loaded prior to loading the qPCR plate with the following (20 μ l each): fifty-six DNA samples at a concentration of 1 ng/ μ l, two replicates of a calibrator at 1 ng/ μ l, a five step 1:4 serial dilution of golden sample DNA starting with 20 ng/ μ l and a negative control (nuclease free water).

A liquid handling robot (Freedom Evo-2 150; Tecan, Mannedorf, Switzerland) was used to transfer both master mixes and the contents of the sample plate in triplicates onto a 384 well plate. The robot mixed 9 μ l of the respective master mix with 1 μ l of the contents of the sample plate per well.

We used the following qPCR protocol on a LightCycler 480 (Roche): 15 min at 95 °C for enzyme activation followed by 50 cycles of 15 s at 95 °C (denaturation), 30 s at 58 °C (primer annealing) and 30 s at 72 °C (signal acquisition). For the production of the melting curve 1 min at 95 °C was followed by 30 s at 58 °C and a continuous increase of 0.11°C/s to 95 °C with continuous signal acquisition. The protocol was finalised by a 10 s cool down at 40 °C.

3.6.6. qPCR quality control

The software package LinReg PCR (Ruijter et al. 2009) was used for a baseline-correction of the amplification curves, the calculation of the plate and reaction specific qPCR efficiency, and the calculation of C_q values. The plate and reaction specific efficiency was calculated by averaging across all well-specific efficiencies of the respective reaction (B2M or telomere reaction) in each plate, after excluding the top and bottom 5%.

The reaction specific efficiencies for all 25 qPCR plates ranged from 94.3% to 94.85% for the B2M reaction and from 91.5%-95.85% for the telomere reaction.

For the calculation of C_q values, thresholds were set for all plates within the window of linearity; thresholds were 0.221 for the B2M reaction and 0.256 for the telomere reaction.

Individual samples failed the quality control if the C_q values across their triplicate varied too much (CV >5%). They also failed if the efficiency for at least one copy within the triplicate was 5% higher or lower than the respective mean plate efficiency (across the B2M or telomere reaction).

We ran two triplicates of the calibrator sample on each plate, one located in the middle of the plate and the other at its periphery. For the calibrator sample, Cq values and efficiencies had to meet our quality control criteria calculated over all six wells (both triplicates) per reaction to ensure high intra-plate repeatability.

To test the accuracy of our qPCR assay, we repeated the measurement of an identical qPCR plate four times over two days. Correlation coefficients for all possible plate combinations were high ($r = 0.75-0.89$) which implies that the rank order of samples stayed similar over all measurements. We fitted a mixed linear model with qPCR plate as fixed effect and sample ID as random effect and calculated a repeatability (variance due to the sample divided by total variance) of 80%.

Samples that failed the post-qPCR quality control (26 in total; 1.55%) were measured again and all passed quality control at the second attempt.

3.6.7. RLTL calculation

RLTL was calculated using following formula published by Pfaffl (2001):

$$(1) \quad RLTL = \frac{E_{TEL}^{Cq_{TEL}(\text{Calibrator}) - Cq_{TEL}(\text{Sample})}}{E_{B2M}^{Cq_{B2M}(\text{Calibrator}) - Cq_{B2M}(\text{Sample})}}$$

where E_{TEL} and E_{B2M} are the reaction and plate specific qPCR efficiencies, Cq is the number of cycles after which the amplification curve crosses the set fluorescence threshold, $Cq_{TEL(\text{Calibrator})}$ and $Cq_{B2M(\text{Calibrator})}$ are the Cq values for the calibrator sample for the respective reaction and $Cq_{TEL(\text{Sample})}$ and $Cq_{B2M(\text{Sample})}$ are the Cq values for the respective reaction of the individual sample.

3.6.8. Statistical analysis

All RLTL measurements were log transformed to achieve normal distribution (Shapiro-Wilk normality test: $W = 0.9985$, $p\text{-value} = 0.2988$ and $W = 0.9949$, $p\text{-value} = 0.4604$ for the cow and the calf datasets respectively).

We used mixed models to assess the effect of various factors (including age) on RLTL and to calculate variance components and genetic parameters. We started by fitting a basic model including the animal identity as a random effect and qPCR plate and the sample position on the qPCR plate (row) as fixed effects. Our previous work has shown that the calibrator sample is not sufficient to completely correct for the

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measurement error that is associated with the qPCR plate (Seeker et al. 2016) and therefore we needed to correct for it statistically. Position effects in qPCR assays have also been observed to cause measurement error in previous studies (Eisenberg et al. 2015; Kibriya et al. 2016). We found in a preliminary test with one identical sample repeated in all wells that the best way to adjust for the well effect is by fitting the row in which the sample was located in the model as a fixed effect.

Next we investigated RLTL dynamics with age by comparing models that incorporated age fitted in different ways in addition to the factors of the basic model. Linear, quadratic, cubic and quartic polynomial functions of age were fitted and assessed based on their AIC values, because models were non-nested (Table 2). Low AIC values were desirable. Within the range of two units (which is corresponding to an approximate significance of $p < 0.05$) the simpler model was preferred over the more complicated (Burnham & Anderson 2002; Arnold 2010; Froy et al. 2015). Because of a distinct difference in RLTL measurements between neonates and older animals in the cow dataset, we tested age as a factor with two levels. We computed separate models for all possible points of separation between the age groups (age in months) and then selected the age cut-off that minimised the residual sum of squares (Table S3). For consistency the same procedure was applied to the calf dataset (Table S4). Once we were satisfied with the age term in our models, other factors that might affect RLTL were tested. For the cow dataset we examined the animal's genetic and feed group, the birth year and the birth season as fixed effects and the animal's birth weight as covariate. We also included all possible interactions between main effects. We were specifically interested in the interaction of age with the animal's birth weight, birth year and birth season, because we suspected that those effects might influence RLTL shortly after birth, but probably not later in life. All non-significant factors and interactions ($P > 0.05$) were backwards eliminated. The effect size of the age factor in the cow dataset was estimated by calculating Cohen's D as the mean difference between factor level estimates divided by the standard deviation. The standard deviation was calculated as the product of the standard error and the square root of the sample size. For the calf dataset we examined the following fixed effects: a quadratic function of age in days, the genetic group of the animal, birth weight, birth year, and birth season. Feed group was not tested, because animals of the calf group were too young to have been allocated to a feeding group; the latter usually takes place when the cow

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gives birth for the first time at about two years of age. We backwards eliminated all non-significant fixed effects with a p-value greater than 0.05. For both datasets, the investigation of the best fitting function of age was repeated with all significant fixed effects in the model.

The final models were used to estimate variance components and genetic parameters based on the principle of restricted maximum likelihood. Animal pedigree information was fitted to the random animal effect enabling the estimation of genetic variance for RLTL. The pedigree included 11,003 animals spread over 27 generations. The animals in the cow dataset were offspring of 40 sires and 241 dams. The animals in the calf dataset were offspring of 7 sires and 35 dams. A random permanent environment effect was also fitted to account for repeated measures within individual animal. The sum of the genetic, permanent environment and residual variances yielded estimates of the total phenotypic variance of RLTL. The ratio of genetic to total phenotypic variance was used to estimate the narrow-sense heritability of the trait. The ratio of the sum of genetic and permanent environment to the total phenotypic variance was used to estimate the repeatability of the trait. The ASReml software (Gilmour et al. 2009) was used for the estimation of variance components and calculation of their ratios.

Next, we investigated the association between RLTL and lifespan measurements. We defined “productive lifespan” as the age in days of the animal at culling and functional longevity as productive lifespan corrected for milk yield. To assess the relationship between functional longevity and RLTL we fitted linear models with productive lifespan (normal distributed, Shapiro-Wilk normality test: $W = 0.9889$, $p = 0.31$) as response variable and the average milk yield calculated over the cow’s lifetime as fixed effect. We added RLTL measurements that were pre-adjusted for qPCR plate and row as second fixed effect to the model. Because animals had to have both productive lifespan and productivity measurements to be included in the analysis, estimations were based on 143 animals for RLTL at birth and on 136 animals for RLTL at the age of one year only.

We investigated the possibility of analysing productive lifespan as a proxy for functional longevity by testing their correlation, because the analysis of the former would allow us to include animals that died before providing a productivity measurement in our calculations. Also, we introduced a censoring group to account

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for animals that were still alive and therefore had no productive lifespan measure yet. We used Cox proportional hazard models for the subsequent analyses. These measures increased the sample size to 305 at birth and to 53 at the age of one year. With the Cox proportional hazard analysis we tested the subsequent survival time after sampling at the ages of 0, 1, 2, 3, 4 and 5 years. RLTL measurements were pre-adjusted for qPCR plate and row. Significance of RLTL at test all test ages on productive lifespan was estimated using the Wald test. To visualise the association of TL at the age of one year with survival probability we repeated the Cox proportional hazard analysis using TL tertiles at this age.

If not stated otherwise, statistical analyses were performed in R studio with R 3.1.3.(R Core Team 2014). Mixed-effects models were implemented using the 'lme4' library. Cox proportional hazard analysis was conducted using the package 'survival'. The significance threshold for all statistical analyses was set to $\alpha=0.05$.

3.6.9. Data availability

All data are available at <https://github.com/LASeeker/Bovine-telomere-dynamics>

3.7. Acknowledgements

We thank everyone involved in collecting blood samples on the Crichton farm, particularly Hannah Dykes and Isla McCubbin. We also thank Ian Archibald for support with sample and data handling. The work was funded by a PhD scholarship to the first author offered by Scotland's Rural College (<http://www.sruc.ac.uk/>) and the Biotechnology and Biological Sciences Research Council (BB/L007312/1; <http://www.bbsrc.ac.uk/>), and the Rural & Environment Science & Analytical Services Division of the Scottish Government. We thank the scientific reviewers for their thorough reading and helpful comments that improved the manuscript considerably.

3.8. Author contributions

AB collected samples on the farm. L.S, S.U, R.W, JF and R.H extracted DNA and performed quality control checks. Together with E.SC they measured RLTL by qPCR. L.S, S.U, R.W and J.F performed post qPCR quality controls and were

together with J.I responsible for the data curation. The statistical analysis was performed by L.S with support from J.I, A.P and H.F and under supervision of G.B and D.N. L.S visualised data and drafted the first manuscript. G.B and D.N reviewed and edited the first draft. All authors reviewed and edited the final draft. G.B, D.N, M.C and B.W supervised the project and acquired funding. All the authors were involved in project planning. G.B was the project principal investigator.

3.9. Additional information

The authors declare that no competing interests exist.

3.10. Thesis context

The present chapter contributes to addressing the second, third and fourth objectives of this thesis. The dynamics of RLTL with age was explored at a population level and it was found that RLTL declines within the first year of life but remains relatively stable thereafter. Factors that influence RLTL such as birth year, birth season and birth weight were identified. It was demonstrated that RLTL at specific ages is associated with productive lifespan. Also, the first heritability estimates of RLTL in a livestock species were presented. This chapter opens up the way for further work required to understand RLTL dynamics in the individual animal and the relationship between individual RLTL change with age and productive lifespan, which is presented in the following two chapters.

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3.11. Chapter appendix (Supplementary File 1)

Table S1: Allocation of animals to genetic and feeding groups

	Genetic select line	Genetic control line	Total
Cow dataset			
High forage diet	57	53	110
Low forage diet	58	60	118
Not allocated to diet	20	60	80
Total	135	173	308
Calf dataset			
Not allocated to diet	22	16	38

Table S2: Birth years and seasons of animals in the cow and calf datasets

	Cow dataset	Calf dataset
Birth year		
2008	25	/
2009	90	/
2010	89	/
2011	70	/
2012	28	/
2013	4	/
2014	2	36
2015	/	2
Birth season		
1 (April to September)	152	19
2 (October to March)	156	19

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Table S3: Cow dataset: Comparison of models that included age as a factor with two levels (younger vs. older at the given age in months). AIC: Akaike information criterion. The smaller the AIC, the better is the model fit. Results are sorted by the AIC value (first AIC is the smallest).

Age in months	AIC	Age in months	AIC	Age in months	AIC	Age in months	AIC
1	-1625.13	20	-1495.86	36	-1445.49	52	-1436.58
3	-1624.3	21	-1491.87	40	-1445.2	51	-1436.55
4	-1624.3	22	-1491.59	41	-1445.19	59	-1436.51
5	-1624.3	23	-1483.33	42	-1442.93	66	-1436.39
6	-1624.3	24	-1482.06	43	-1441.93	54	-1436.18
2	-1623.71	25	-1475.31	44	-1441.43	53	-1436.17
7	-1615.92	26	-1474.58	45	-1440.83	63	-1436.17
8	-1591.34	27	-1469.37	47	-1439.66	58	-1436.15
9	-1574.94	28	-1467.7	48	-1439.49	60	-1436.06
10	-1554.28	30	-1462.34	46	-1439.31	61	-1436.06
11	-1554.27	29	-1461.71	69	-1437.91	55	-1436.01
12	-1535.6	31	-1457.9	49	-1437.88	56	-1436.01
13	-1525.91	32	-1453.99	68	-1437.69	57	-1435.99
14	-1521.79	33	-1451.78	50	-1437.57	64	-1435.98
15	-1511.8	34	-1449.02	70	-1437.42	62	-1435.98
16	-1500.09	39	-1446.96	71	-1437.23	75	-1435.97
18	-1498.82	35	-1446.84	72	-1437.23	65	-1435.96
17	-1498.57	38	-1446.3	73	-1437.23	67	-1435.96
19	-1496.74	37	-1445.7	74	-1437.23		

Table S4: Calf dataset: Comparison of models that included age as a factor with two levels (younger vs. older at the given age in months). AIC: Akaike information criterion. The smaller the AIC, the better is the model fit. Results are sorted by the AIC value (first AIC is the smallest).

Age in Months	AIC
4	-276.92
3	-273.976
5	-272.586
6	-269.329
2	-263.047
7	-262.209
1	-260.774
8	-257.075
9	-254.115
11	-254.007
10	-253.898

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Table S5: Cow dataset. Fixed effect estimates of the final model. Age was fitted as a two level factor (younger vs. older than one month) and animal ID was fitted as random effect. Variance components are shown in table 2 of the manuscript.

Factor	Estimate	Std. Error	df	t value	p value for factor level	p value for factor
(Intercept)	0.103676	0.028125	744.4827	3.686298	0.000244063	
age over 1 month	-0.10599	0.007469	1012.862	-14.1911	<0.0001	<0.0001
birth year 2009	0.035954	0.023252	275.7098	1.546259	0.123189057	0.004
birth year 2010	-0.02469	0.023353	279.6671	-1.0573	0.291286032	0.004
birth year 2011	-0.00911	0.024263	290.0133	-0.37558	0.707504856	0.004
birth year 2012	-0.02104	0.029774	334.91	-0.70659	0.480312438	0.004
birth year 2013	0.075361	0.063079	460.202	1.194703	0.232818364	0.004
birth year 2014	0.049842	0.094348	599.1338	0.528281	0.597499673	0.004
qPCR plate 2	0.025009	0.023093	1149.339	1.082953	0.27905642	<0.0001
qPCR plate 3	0.021179	0.023263	1157.139	0.910411	0.362795349	<0.0001
qPCR plate 4	0.008526	0.023532	1153.672	0.36229	0.717201957	<0.0001
qPCR plate 5	-0.00539	0.023228	1151.427	-0.2321	0.816504973	<0.0001
qPCR plate 6	-0.00944	0.023302	1158.579	-0.40528	0.685343015	<0.0001
qPCR plate 7	0.01214	0.023483	1147.634	0.516977	0.605272151	<0.0001
qPCR plate 8	-0.00379	0.02302	1138.496	-0.16484	0.869100473	<0.0001
qPCR plate 9	-0.00696	0.023301	1127.711	-0.29876	0.765179091	<0.0001
qPCR plate 10	0.034607	0.023038	1141.303	1.502143	0.133336876	<0.0001
qPCR plate 11	0.013568	0.023145	1156.444	0.586212	0.557847197	<0.0001
qPCR plate 12	0.036181	0.023322	1156.302	1.551387	0.121082682	<0.0001
qPCR plate 13	0.061542	0.023235	1141.643	2.648723	0.008191132	<0.0001
qPCR plate 14	0.046593	0.023119	1139.199	2.015321	0.04410549	<0.0001
qPCR plate 15	-0.00436	0.023591	1161.397	-0.18489	0.853346273	<0.0001
qPCR plate 16	0.017643	0.023437	1151.824	0.752816	0.451714024	<0.0001
qPCR plate 17	0.155652	0.023199	1154.853	6.709526	<0.0001	<0.0001
qPCR plate 18	0.102508	0.023229	1154.692	4.413011	<0.0001	<0.0001
qPCR plate 19	0.052811	0.023273	1131.334	2.269159	0.023446309	<0.0001
qPCR plate 20	0.015264	0.023383	1160.788	0.652755	0.514043213	<0.0001
qPCR plate 21	0.029346	0.023433	1145.858	1.252351	0.210697439	<0.0001
qPCR plate 22	-0.00703	0.022984	1133.92	-0.30599	0.75966473	<0.0001
qPCR plate 23	-0.04198	0.02345	1155.558	-1.79022	0.073681055	<0.0001
qPCR plate 24	-0.01607	0.025524	1153.953	-0.62955	0.529116527	<0.0001
qPCR plate 25	0.032122	0.030063	1128.132	1.068505	0.285521536	<0.0001
qPCR row B	0.11563	0.012884	1136.105	8.974376	<0.0001	<0.0001
qPCR row C	0.067461	0.01279	1125.76	5.274391	<0.0001	<0.0001
qPCR row D	-0.0728	0.012873	1132.07	-5.65538	<0.0001	<0.0001
qPCR row E	-0.00207	0.013454	1141.619	-0.15349	0.878035957	<0.0001
qPCR row F	0.014852	0.012821	1124.566	1.158425	0.246936589	<0.0001
qPCR row G	0.027846	0.012982	1144.541	2.144991	0.032163561	<0.0001
qPCR row H	-0.01398	0.013242	1133.391	-1.05551	0.291416627	<0.0001

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Table S6: Calf dataset. Fixed effect estimates of the final model. A quadratic function was fitted to age in days and animal ID was fitted as random effect. Variance components are shown in table 2 of the manuscript.

Factor	Estimate	Std. Error	df	t value	p value for factor level	overall p value for factor
(Intercept)	0.191865	0.098877	46.111	1.940429	0.058456	
Linear component of age in days	-0.60565	0.137759	242.438	-4.39647	1.65E-05	<0.0001
Quadratic component of age in days	0.293872	0.135379	237.3025	2.170735	0.030943	<0.0001
Birth season 2	0.069303	0.032652	36.3774	2.122456	0.040673	0.040673
Birth weight	-0.00501	0.002414	36.64214	-2.07671	0.044897	0.044897
qPCR plate 2	0.063097	0.027462	246.3607	2.297618	0.022422	<0.001
qPCR plate 3	0.075581	0.027529	245.6671	2.74552	0.006488	<0.001
qPCR plate 4	0.067166	0.027162	243.4212	2.47284	0.014088	<0.001
qPCR plate 5	0.143915	0.027615	247.275	5.211416	3.95E-07	<0.001
qPCR plate 6	0.049379	0.031789	262.1415	1.553343	0.121548	<0.001
qPCR row B	-0.05229	0.032564	249.8263	-1.6057	0.109603	0.001
qPCR row C	0.035326	0.031299	242.5322	1.128638	0.260166	0.001
qPCR row D	0.010171	0.031753	245.8092	0.32031	0.749006	0.001
qPCR row E	-0.00395	0.03201	242.3175	-0.12341	0.901888	0.001
qPCR row F	-0.07199	0.032427	246.2654	-2.22009	0.027324	0.001
qPCR row G	0.057835	0.031049	245.8499	1.86269	0.063699	0.001

Table S7: Reasons of culling in the cow dataset.

Reason of culling	Absolute number of cows	Relative number of cows (%)
fertility problems	77	31.6
mastitis	35	14.3
lameness	34	13.9
productivity	2	0.8
other	96	39.3

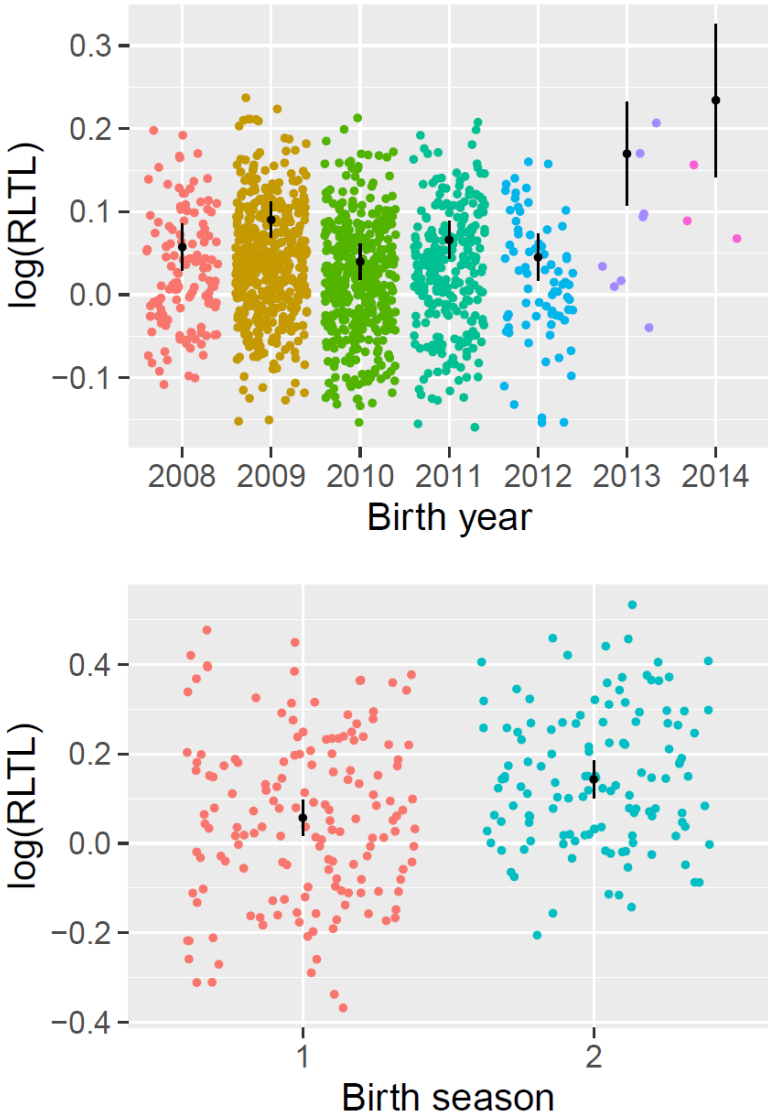


Figure S1: Impact of birth year (cow dataset) and birth season (calf dataset) on RLTL. Model predictions and standard errors are plotted over raw data.

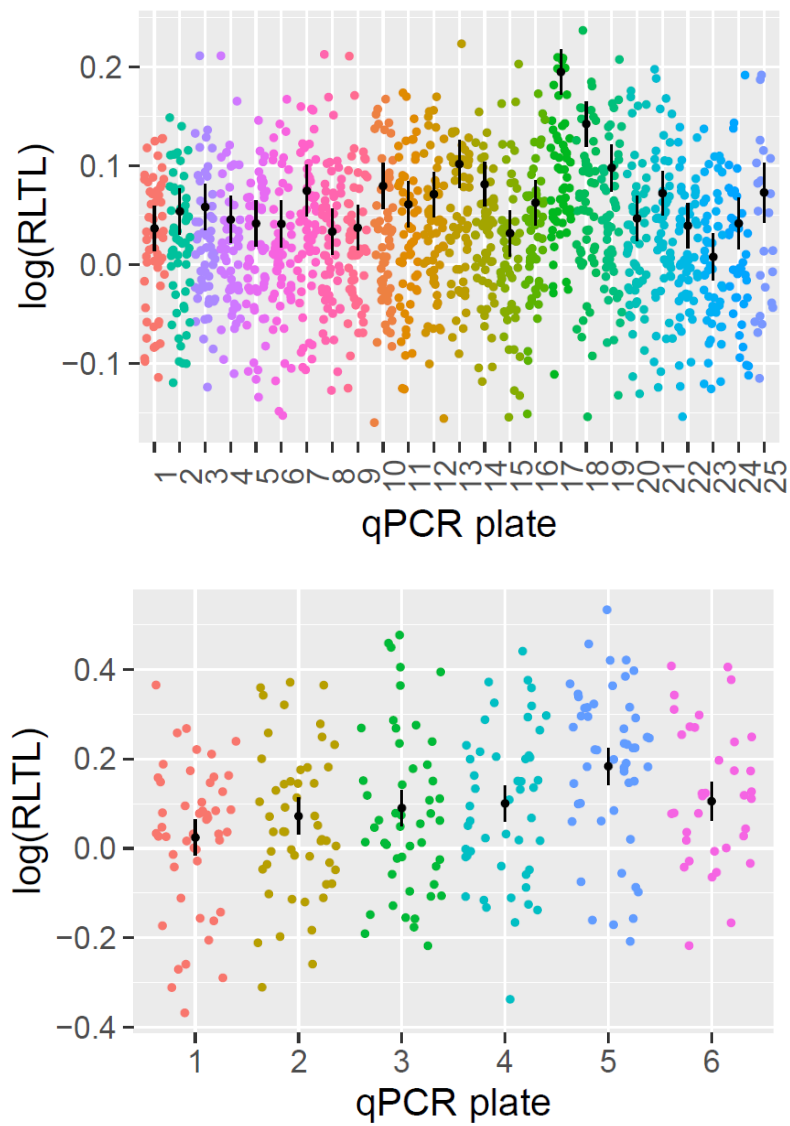


Figure S2: Impact of qPCR plate on RLTL. Model predictions and standard errors are plotted over raw data. Top panel: cow dataset, bottom panel: calf dataset.

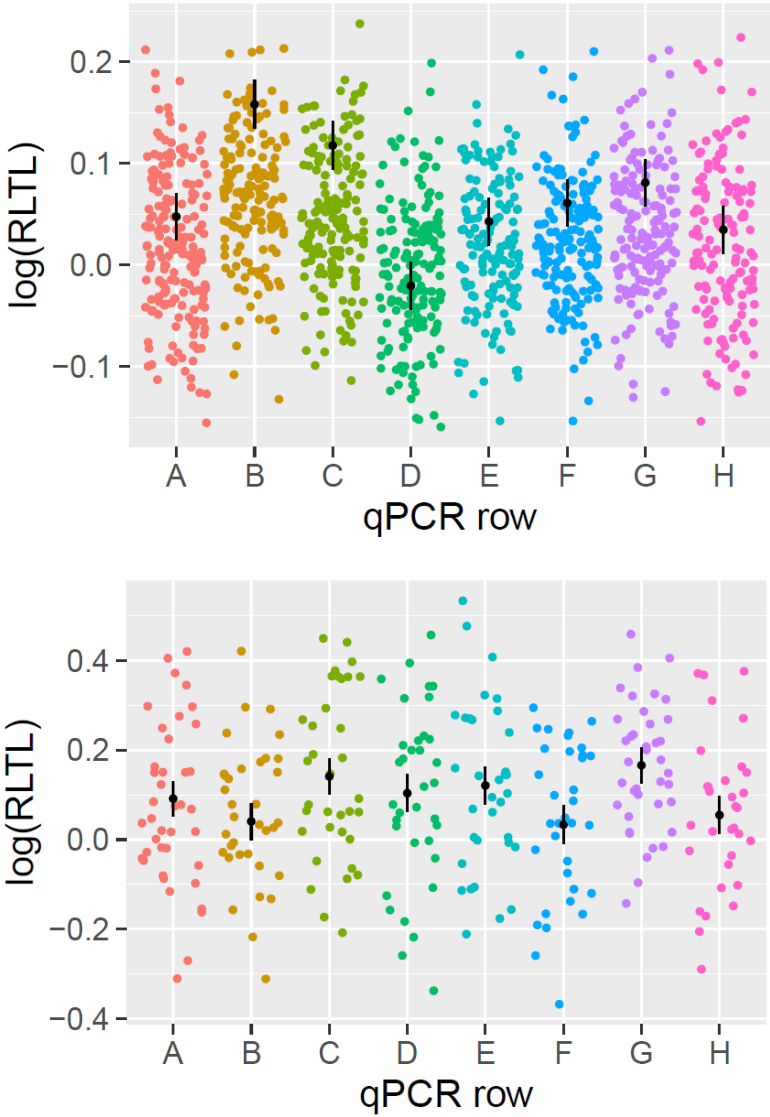


Figure S3: qPCR row effect. Model predictions with standard errors are plotted over raw data. Top panel: cow dataset, bottom panel: calf dataset.

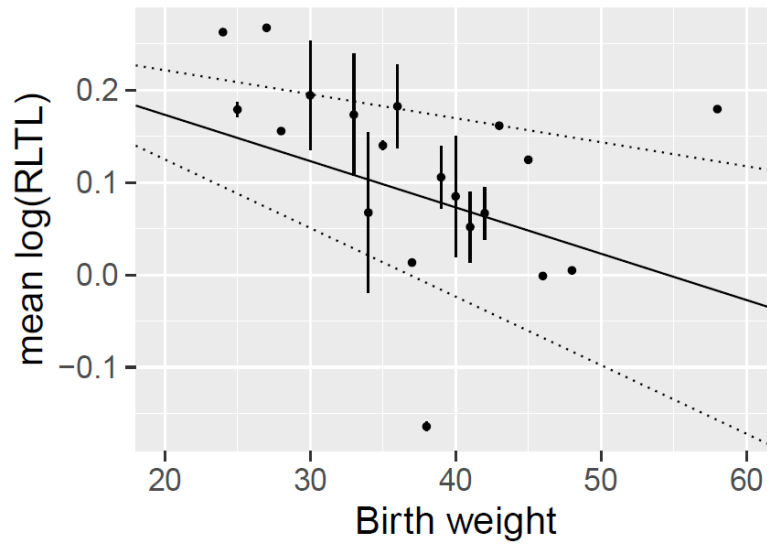


Figure S4: Predicted regression of relative leukocyte telomere length (RLTL) on birth weight. Average raw data with standard errors are shown. The predicted linear equation was $y = -0.005x + 0.274$ ($p = 0.045$).

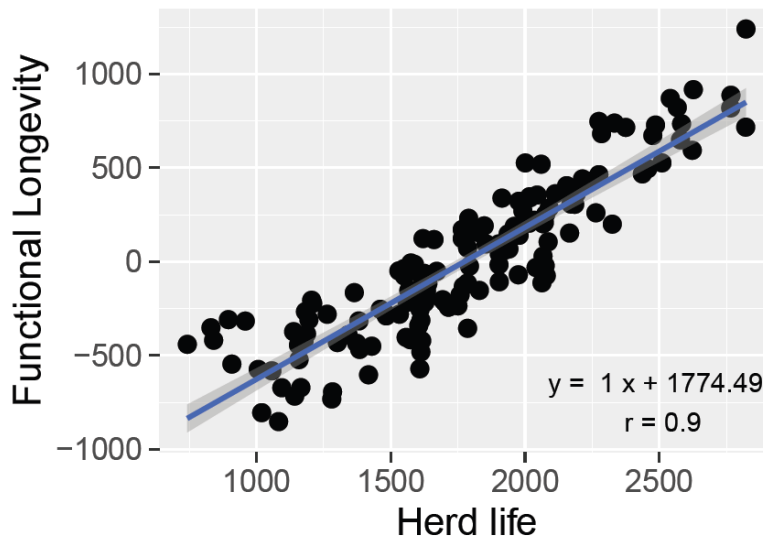


Figure S5: Correlation of functional longevity with productive lifespan ($p < 0.001$).

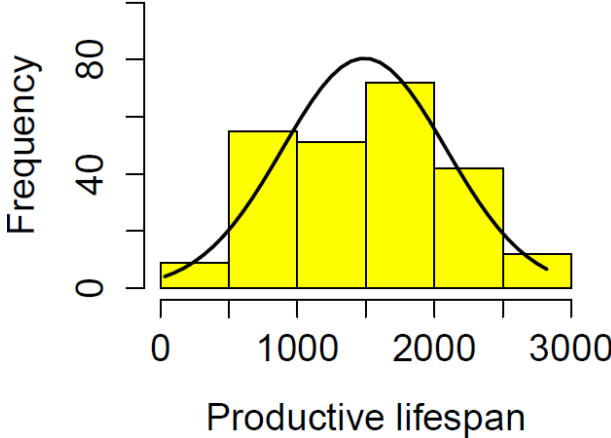


Figure S6: Productive lifespan (=age at culling) in the cow dataset.

Chapter 4:

Longitudinal changes in telomere length and associated genetic parameters in dairy cattle analysed using random regression models

4.1. Prefix

While the previous chapter of this thesis investigated RLTL dynamics at a population level and the association of RLTL with productive lifespan, this chapter aims to describe individual animal RLTL dynamics with age. Also, an association of different RLTL profiles with productive lifespan will be tested. The genetic analysis of RLTL is extended to better understand changes in heritability over life and inter trait correlations.

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I conducted the experimental and analytical work under guidance from the supervisors and wrote the manuscript with the help of supervisors and co-authors.

4.2. Abstract

Telomeres cap the ends of linear chromosomes and shorten with age in many organisms. In humans short telomeres have been linked to morbidity and mortality. With the accumulation of longitudinal datasets the focus shifts from investigating telomere length (TL) to exploring TL change within individuals over time. Some studies indicate that the speed of telomere attrition is predictive of future disease. The objectives of the present study were to 1) characterize the change in bovine relative leukocyte TL (RLTL) across the lifetime in Holstein Friesian dairy cattle, 2) estimate genetic parameters of RLTL over time and 3) investigate the association of differences in individual RLTL profiles with productive lifespan. RLTL measurements were analysed using Legendre polynomials in a random regression model to describe TL profiles and genetic variance over age. The analyses were based on 1,328 repeated RLTL measurements of 308 female Holstein Friesian dairy cattle. A quadratic Legendre polynomial was fitted to the fixed effect of age in months and to the random effect of the animal identity. Changes in RLTL, heritability and within-trait genetic correlation along the age trajectory were calculated and illustrated. At a population level, the relationship between RLTL and age was described by a positive quadratic function. Individuals varied significantly regarding the direction and amount of RLTL change over life. The heritability of RLTL ranged from 0.36 to 0.47 (SE= 0.05-0.08) and remained statistically unchanged over time. The genetic correlation of RLTL at birth with measurements later in life decreased with the time interval between samplings from near unity to 0.69, indicating that TL later in life might be regulated by different genes than TL early in life. Even though animals differed in their RLTL profiles significantly, those differences were not correlated with productive lifespan ($p=0.954$).

4.3. Introduction

Telomeres are located at the ends of linear chromosomes. They consist of non-coding nucleotide tandem repeats (TTAGGG in vertebrates) and attached proteins of the shelterin complex (Blackburn & Gall 1978; De Lange 2005; Moyzis et al. 1988). Since telomeres were first shown to shorten with the number of cell divisions in vitro (Harley, Futcher & CW. Greider 1990), they have been intensely studied in relation to ageing and lifespan in various species in vivo (Bakaysa et al. 2007; Haussmann & Marchetto 2010; Haussmann et al. 2005; Herborn et al. 2014; Monaghan & Haussmann 2006). Such studies have reported mixed results. While

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some observed a positive correlation between telomere length and longevity (Bakaysa et al. 2007; Boonekamp et al. 2013; Fairlie et al. 2015; Heidinger et al. 2012), others found no relationship (Hovatta et al. 2012; Raymond et al. 2012). Many authors claimed that longitudinal studies were necessary to better understand telomere dynamics within the individual, and to investigate the association of not only telomere length but also change in telomere length with lifespan (Boonekamp et al. 2013; Hornsby 2006; Lansdorp 2006; Mather et al. 2011). In longitudinal studies of Alpine swifts and Seychelles warblers, faster telomere attrition, but not telomere length per se, was associated with poorer survival (Barrett et al. 2013; Bize et al. 2009). In humans telomere length maintenance was associated with better survival than telomere length attrition in patients with cardiovascular disease (Farzaneh-Far et al. 2010; Goglin et al. 2016). However, the relationship between telomere length attrition and survival has not been investigated in a livestock species to date.

Genetic studies on telomere length are rare outside the human literature. In humans it has been shown that telomere length is a quantitative trait that is controlled by many different loci (Pooley et al. 2013; Gatbonton et al. 2006; Levy et al. 2010; Shen et al. 2011; Mangino et al. 2008). Heritability estimates are available for humans, sand lizards and kakapos and range from 0.39 to 0.82 in those species (Vasa-Nicotera et al. 2005; Bischoff et al. 2005; Broer et al. 2013; Hjelmborg et al. 2015; Njajou et al. 2007; Olsson, Pauliny, Wapstra, Uller, Schwartz & Blomqvist 2011; Horn et al. 2011). Outside those studies heritability estimates are missing from the literature. It has been shown in the above mentioned species that telomere length is a heritable trait, but it is unclear if heritability estimates change over life or are relatively constant. A changing impact of environmental effects on telomere length might change heritability estimates over time. For animal breeders it is interesting to know which proportion of a trait at any time is caused by genetic effects and therefore possible to influence with breeding.

In the livestock sector there is a growing interest in using telomere length as a biomarker for health, productive lifespan and animal welfare (Bateson 2016; Brown et al. 2012). However, longitudinal studies that investigate change in telomere length within individuals are largely missing from the livestock literature. In the present study we are interested in the rate and direction of telomere length change and the relationship of different telomere length change profiles with productive lifespan. We

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use random regression models which were initially developed to describe lactation curves in dairy cattle (Jamrozik & Schaeffer 1997; Kirkpatrick et al. 1994) for the analysis of telomere length profiles. They allow the fitting of an overall fixed curve across time which describes the population trend, and individual random animal curves (profiles) as deviations from the former. Random regression models take into account the correlation among repeated measurements within an individual, which is usually greater than the correlation of measurements between animals (Werf 2001). Over the last two decades random regression models have been applied to many studies in genetics and evolutionary ecology addressing the change of a broad range of traits over time. Examples of studied traits in genetics include milk yield (Mrode & Coffey 2008), milk fat and protein content (Abdollahpour et al. 2013), somatic cell count (Mrode & Swanson 2003), body condition score (Banos et al. 2004; Oikonomou et al. 2008), body energy (Banos & Coffey 2010) and carcass traits (Englishby et al. 2016). In evolutionary ecology studied traits included fitness (Nussey et al. 2008), body size (Wilson et al. 2007), body weight in relation to faecal egg counts (Hayward et al. 2014) and antler size (Kruuk et al. 2002). To our knowledge, only a single study has used random regression models for the analysis of longitudinal telomere data so far (Barrett et al. 2013). However, the study was based on a rather small dataset (373 samples of 204 individuals; more than half of the individuals were sampled once only) and could not find a statistically significant difference in telomere length profiles.

The objectives of the present study were to 1) characterize the change in bovine relative leukocyte telomere length (RLTL) across the lifetime in Holstein Friesian dairy cattle, 2) estimate genetic parameters of RLTL over time and 3) investigate the association of differences in individual RLTL profiles with productive lifespan.

4.4. Materials and Methods

4.4.1. Ethics statement

Blood sampling of Holstein Friesian cattle was approved by the Animal Experiments Committee (UK Home Office Project License Number: PPL 60/4278).

4.4.2. Data

Animals used in this study were Holstein Friesian dairy cattle of the Langhill herd that were kept at the Crichton Royal Research Farm in Dumfries (Scotland, UK). All animals in this herd belong to one of two distinct genetic lines (selected for high milk

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fat and protein yield vs. control). Furthermore, cows are randomly allocated to two different diets that contain either a high or low proportion of forage. These genetic lines and diets were set up over 30 years ago to accommodate genetic and nutritional scientific studies (Veerkamp et al. 1994).

We measured RLTL in 1,328 longitudinal samples of 308 female animals born between 2008 and 2014. Animals were approximately equally split between genetic lines and diets. All animals were blood sampled once at birth and then at least once more during their lifetime. On average, 4.3 samples were taken per animal. At the end of the study 244 out of 308 animals were dead and had recorded productive lifespan measurements. Productive lifespan was defined as the time between the animal's birth and culling in days. Productive lifespan differs from longevity measurements in humans and natural populations, because dairy cattle rarely die of natural causes. However, we argue that productive lifespan is still biologically meaningful, because animals are not culled randomly but usually for fertility or health reasons.

DNA was extracted from whole blood samples using DNeasy spin columns (QIAGEN) and each sample had to pass internal quality control steps which were 1) yield and purity measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) had to fulfil the minimum requirements of: yield > 20 ng/μl, 260/280 ratio > 1.7 and 260/230 ratio >1.8 and 2) integrity gel scores had to be between 1-2 (Seeker et al. 2016). RLTL was measured by qPCR using 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') primers (Epel et al. 2004) for the telomere amplification and beta-2-microglobulin (B2M) primers (Primerdesign, accession code NM_001009284) for the reference gene amplification (Seeker et al. 2016). An identical sample – the so-called calibrator or golden sample – was repeated on every plate to correct for measurement error that is associated with the qPCR plate. The number of cycles at which the qPCR amplification curve crosses a set fluorescence threshold (the C_q value) was determined for each sample for telomere and B2M reactions. Raw C_q measurements were baseline corrected using the software LinReg PCR (Ruijter et al. 2009). The same software was used to calculate the reaction specific qPCR efficiencies E_{TEL} and E_{B2M} that were in turn used in following formula (Pfaffl 2001) to calculate RLTL:

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$$RLTL = \frac{E_{TEL}^{Cq_{TEL}(\text{Calibrator}) - Cq_{TEL}(\text{Sample})}}{E_{B2M}^{Cq_{B2M}(\text{Calibrator}) - Cq_{B2M}(\text{Sample})}} \quad (1)$$

The Cq values corresponding with the calibrator sample were $Cq_{TEL(\text{Calibrator})}$ and $Cq_{B2M(\text{Calibrator})}$ for the telomere and the B2M reaction respectively. Cq values of the individual samples were $Cq_{TEL(\text{Sample})}$ and $Cq_{B2M(\text{Sample})}$.

Individual samples were measured on 25 qPCR plates in total which had 8 rows for each reaction. RLTL data were logarithmically transformed to achieve normal distribution (Shapiro-Wilk normality test: $W = 0.9985$, $p = 0.299$). Because of the increasing scarcity of data points after the age of 60 months, this age was used as the cut-off for data visualisation. The pedigree included 11,003 animals spread over 27 generations. The animals with RLTL measurements were descendants of 40 sires and 241 dams.

4.4.3. Data analysis

The following random regression model was used for the analysis of longitudinal RLTL data:

$$Y_{tijk} = \text{BirthYear}_j + \text{GeneticGroup}_j + \text{qPCRplate}_{ij} + \text{qPCRrow}_{ij} + \sum_{k=0}^n P_{jkt} b_k + \sum_{k=0}^n P_{jkt} u_{jk} + e_{tijk} \quad (2)$$

where Y_{tijk} = the i^{th} RLTL measurement for animal j using a Legendre polynomial of the order k . BirthYear_j represents the fixed effect of the year in which animal j was born; GeneticGroup_j stands for the fixed effect of the genetic group of animal j ; qPCR plate and qPCR row of a particular sample i of animal j was included as fixed effects (qPCRplate_{ij} and qPCRrow_{ij}); fixed effects regression coefficients are represented by b_k , while u_{jk} stands for the k^{th} order random regression coefficients for the additive genetic effects of animal j ; P_{jkt} represents the k^{th} order of Legendre polynomial fitted to the measurement i of animal j at the age t in months; the random residual variance is e_{tijk} . Sampling intervals and age at sampling (after the initial record) differed among individuals.

Model (2) included fixed effects that remained statistically significant ($p < 0.05$) after backwards eliminating all tested non-significant effects (such as birth season, birth weight, weight at sampling, body condition score and feed group) and the genetic

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group of the animal. The fixed and random regressions, both modelled with polynomial functions, described the average RTL change across age, and individual animal deviations from the average, respectively. The latter pertained to the animal's additive genetic effect. The animal's permanent environment was also examined as a random factor but had a negligible effect (see S1 File).

We tested if the residual variance of different age groups differed significantly implying a heterogeneous variance structure. We first considered four different age groups (0-12 months, 13-24 months, 25-40 months and older than 40 months) and then two different age groups (younger and older than 2 months) but did not find a significant difference in residual variance between any age groups (see S1 File). Therefore, a homogeneous residual variance structure was assumed for the subsequent analysis.

The Akaike information criterion (AIC) was used to assess 1) if the introduction of the random animal genetic effect improved the model fit compared to a model that only included fixed effects; this would suggest that animals differ in their intercept (average RLTL across all measurements); 2) if Legendre polynomials fitted to the random animal genetic effect improved the model fit further, thereby suggesting that animals also differ in their slope (RLTL dynamics). A difference of two units in AIC corresponds to an approximate significance of $p < 0.05$. Within the range of two units the simpler model was preferred over the more complicated (Burnham & Anderson 2002; Arnold 2010; Froy et al. 2015). In the end, quadratic polynomials were fitted to both the overall fixed curve and the individual random animal deviation.

All statistical analyses were conducted with the ASReml software version 4.1 (Gilmour et al. 2009).

4.4.4. Calculation of the fixed and random curves

The fixed curve that illustrates RLTL dynamics at a population level was calculated as the sum of the products of the Legendre polynomial order residuals for a given age and the corresponding fixed regression coefficients. This was repeated across all ages on the trajectory. Random regression models allow the calculation of an individual profile of RLTL change over age for each animal as a deviation from the population mean (fixed curve). The model output provides estimates (solutions) for each animal and each order of polynomial fitted in the model. The random curves were calculated simply by summing solutions for each animal and test month across

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all products of the n^{th} order polynomial with the n^{th} order polynomial residual. The standard error was calculated in parallel by using the standard errors associated with the solutions for the same calculation. Eigenvalues were calculated to estimate the amount of variance between animals that is due to 1) the intercept and 2) the shape of individual curves. Eigenfunctions were calculated to analyse the direction of each effect.

4.4.5. Variance components and genetic parameters

The additive genetic variance (V_A) for each month was calculated using following formula (Werf 2001):

$$V_A = pKp' \quad (3)$$

Where p is a $1 \times k$ vector (k is the order of the fitted Legendre polynomial) containing the residuals for each polynomial order for the given month, K is a matrix containing the REML estimates of (co)variance components and p' is the transposed p vector. The heritability of RLTL and its standard error were calculated at birth and for each consecutive month. Also, the genetic correlations of RLTL at birth with each following month were calculated. Detailed information about those calculations can be found in S1 File.

4.4.6. Analysis of the association of RLTL dynamics with productive lifespan

Out of 308 animals 244 were dead by the end of the study and produced exact productive lifespan measurements. To investigate if different RLTL profiles were associated with a difference in productive lifespan, individual RLTL random curves (profiles) were clustered using the R library `kmlShape` (Genolini et al. 2016) in five groups. We decided for five clusters to explore a difference in animals that maintain their RLTL in contrast to those who early in life either mildly or moderately shorten or elongate their RLTL, respectively. The association between productive lifespan and RLTL cluster was investigated with a Cox proportional hazard analysis. This analysis allows fitting maximal known survival times as right-censored data to account for animals that are still alive. For living animals age in days at the first day of the present year was used for the calculation of the maximal known survival time. A Wald test was used to determine the significance of the relationship between RLTL profiles and productive lifespan.

4.5. Results

Raw RLTL measures ranged from 0.693 to 1.727 with a mean of 1.082. The coefficient of variation was 0.162. The model that included the animal identity as a random effect fitted the data significantly better than a model including only the fixed effects ($\Delta \text{AIC} = 204.97$) suggesting that animals differed significantly in their average RLTL across time. Fitting animal identity with pedigree information further improved the model fit ($\Delta \text{AIC} = 55.46$). Fitting an individual curve for each animal (using a quadratic Legendre polynomial) additionally increased the model fit ($\Delta \text{AIC} = 3.24$), meaning that monthly RLTL dynamics also differ among individual animals. A quadratic Legendre polynomial fitted marginally better than a linear function ($\Delta \text{AIC} = 2.07$) and had the advantage that the same order of Legendre polynomial was fitted to the fixed and the random effect which facilitates interpretation of the results.

The fixed curve as described by the Legendre polynomial captured the expected initial decline of RLTL in early life and a relative stability of RLTL later in life (Fig 1). The curve also illustrates a slight increase of RLTL in later life.

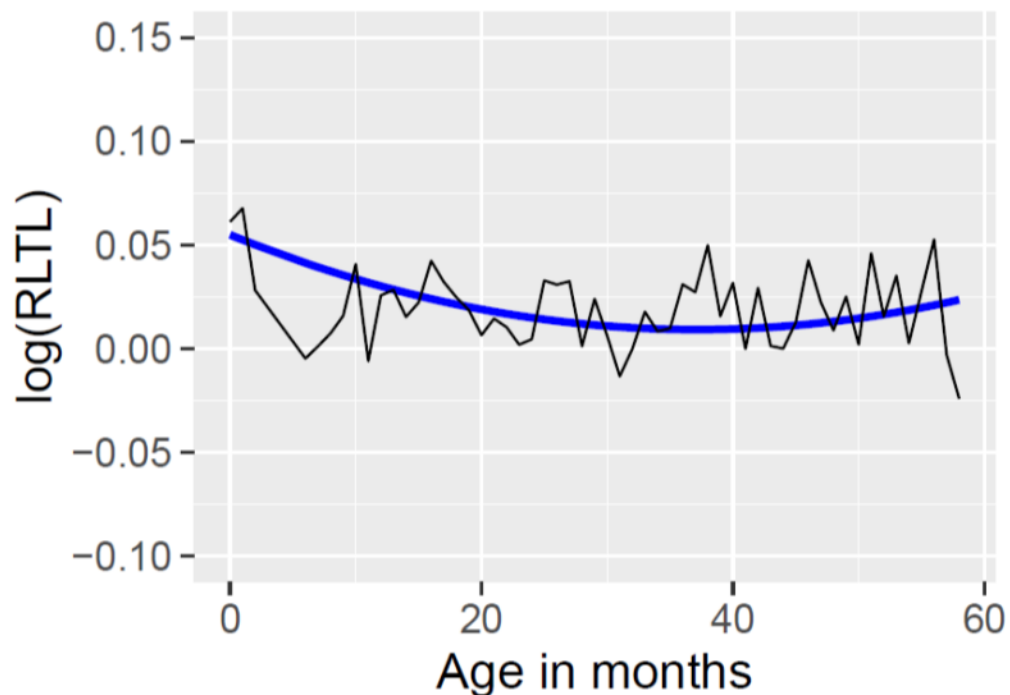


Figure 1: Fixed curve of logarithmically transformed relative leukocyte telomere (RLTL) data. Blue line: quadratic Legendre polynomial function of age; black line: phenotypic RLTL measurements for each month.

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Examples of individual animal RLTL curves are shown in Fig 2. These curves illustrate the change in RLTL with age. The intercept, amount and direction of individual RLTL profiles varied considerably and significantly among the animals in the study (Fig 2). The calculation of eigenvalues revealed that the majority of the difference between individual animal profiles is explained by differences in the intercept (94.7%) while 5.3% are due to different shapes of the curves. Eigenfunctions are shown in S1 File.

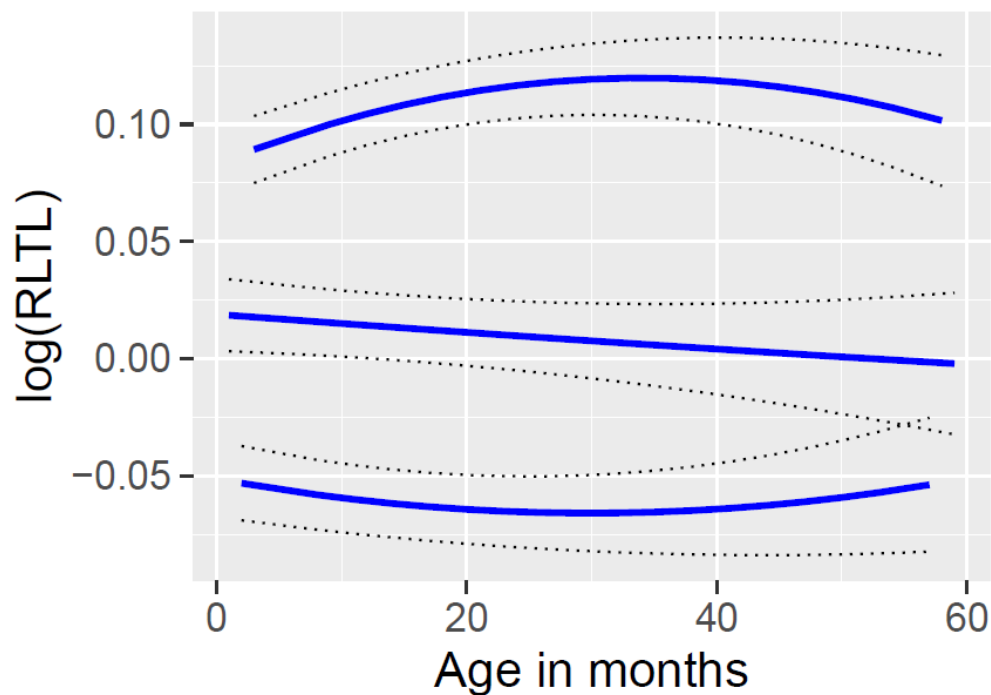


Figure 2: Examples for three individual animal RLTL curves (blue lines) with standard error (black, dotted lines).

Monthly heritability estimates for RLTL ranged from 0.356 to 0.470 (SE= 0.045-0.104) and were slightly higher between 20 and 50 months of age than in the beginning of life or at older ages. Considering the SE, heritability estimates remained relatively stable over life (Fig 3).

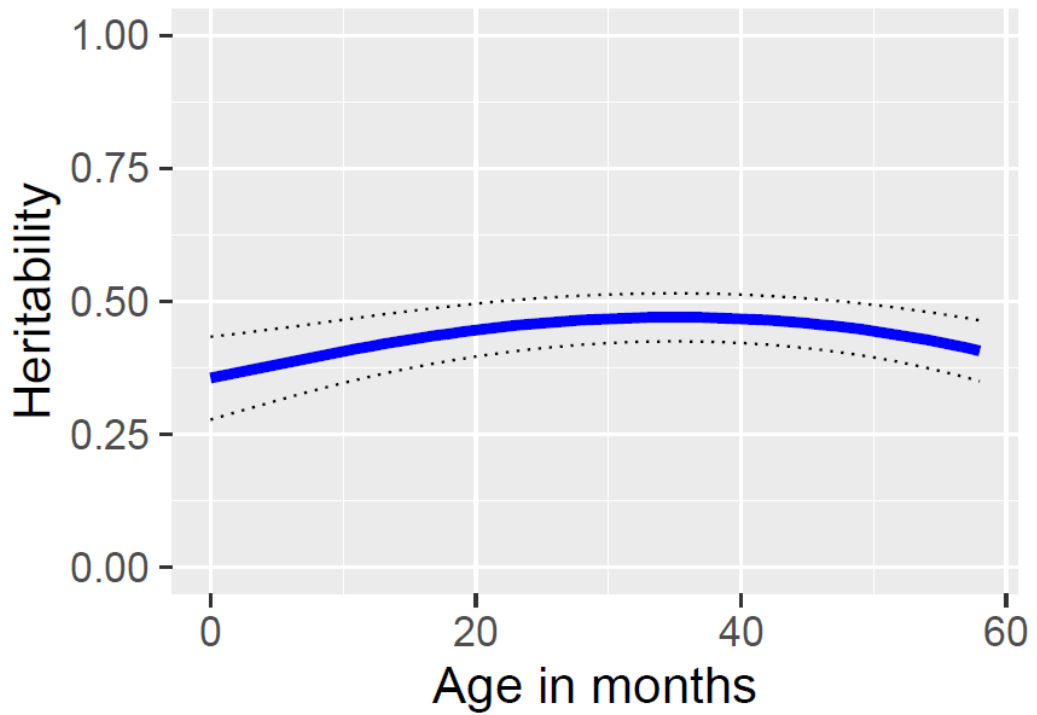


Figure 3: Heritability estimate of RLTL by month of age; standard errors in dotted lines (SE=0.045-0.078).

The genetic correlation between RLTL measurements at birth and at different stages of the animals' lives are shown in Fig 4. As expected, correlations were very high between RLTL at birth and neighbouring ages but decreased as the interval between the two measurements increased. The minimum correlation was 0.693.

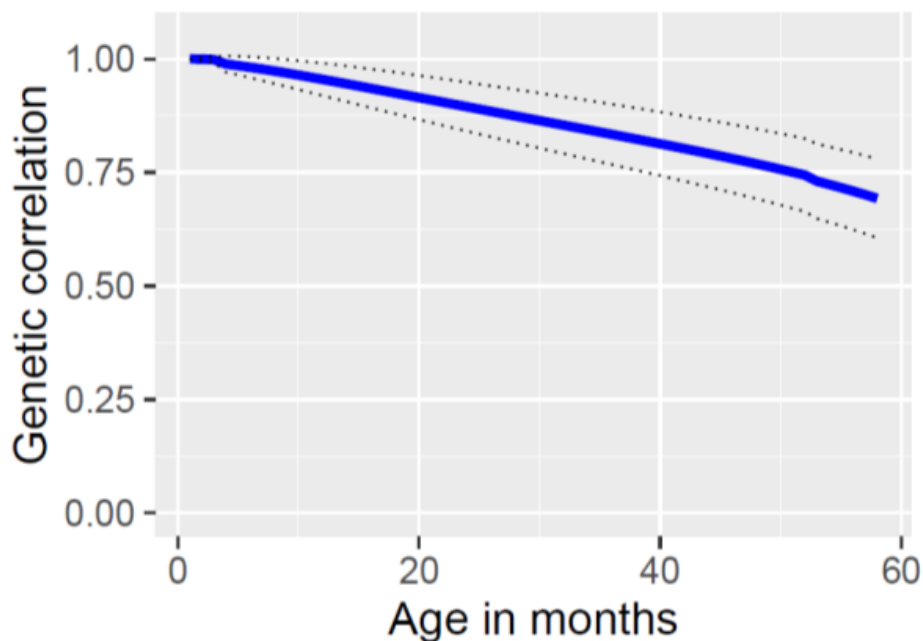


Figure 4: Genetic correlation of RLTL measurements at birth with measurements in later life; standard errors in dotted lines (maximal SE = 0.087).

4.5.1. Analysis of the association of RLTL dynamics with productive lifespan

Productive lifespan ranged from 17 to 2,823 days (mean = 1,477 days, sd= 76.97 days). To test the association between RLTL profiles (intercept and shape) and productive lifespan, RLTL profiles were clustered into groups depending on the similarity of their RLTL change pattern. Five clusters were formed to capture no telomere change and mild and moderate changes in both directions early in life (attrition vs. elongation). Animals differed more in their intercept than in their direction and amount of change. Of all animals 32 % shortened their RLTL slightly in early life, while 29 % did not show obvious RLTL change at all (red curve and green curves respectively in Fig 5). Mild elongation early in life was observed in 22 % (blue curve in Fig 5). More obvious attrition and elongation early in life was observed in 12 % and 5 % of the animals, respectively (cyan and pink curves in Fig 5). The Cox proportional hazard analysis revealed that there was no significant relationship between RLTL profile cluster and productive lifespan ($p=0.97$) which is visualised in Fig 6.

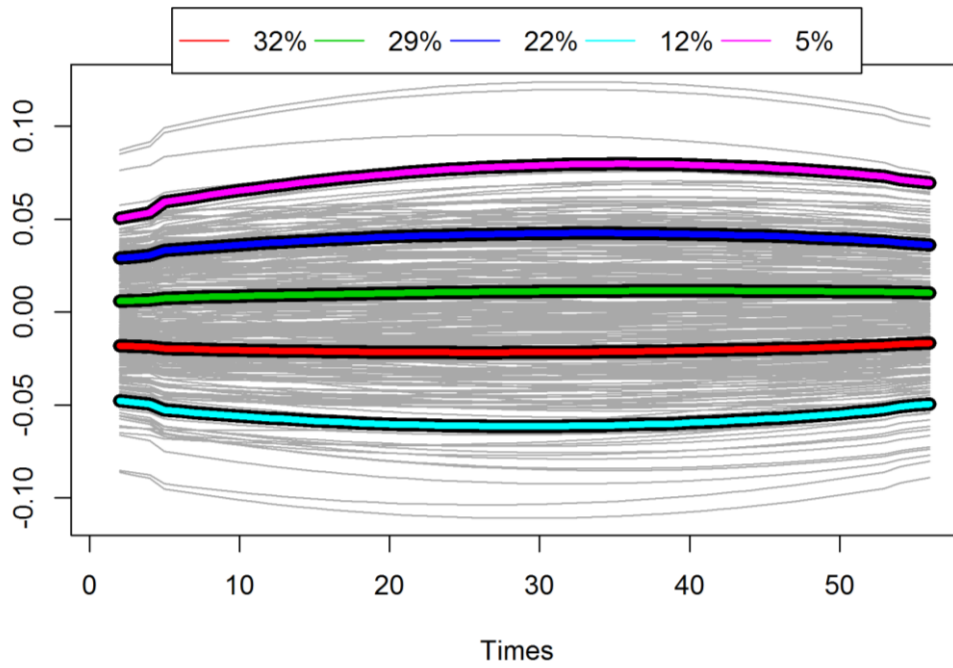


Figure 5: Individual RLTL profiles (grey) and five cluster curves. Of all animals 32 % shortened their RLTL slightly in early life (red curve), 29 % maintained their RLTL over life (green curve), 22 % showed mild elongation in early life (blue curve), 12 % more obvious elongation (pink curve) and 5 % more obvious telomere attrition (cyan curve).

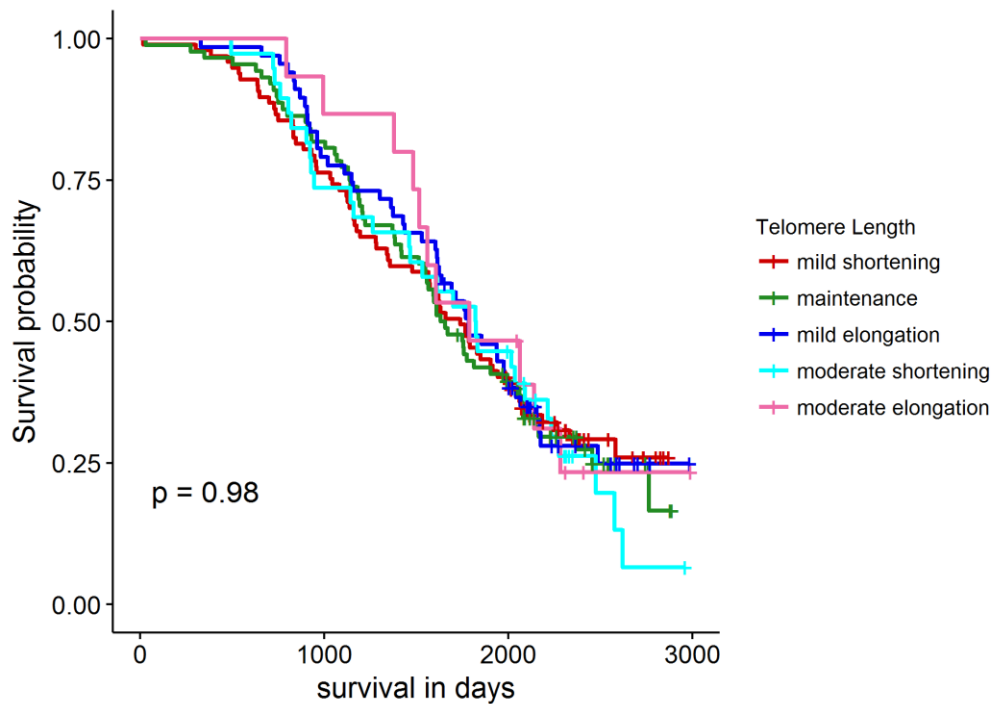


Fig 6: Survival probability of different RLTL profile cluster groups. Colours correspond to colours in Fig 5.

4.6. Discussion

This is the first study exploring individual RLTL profiles of farm animals across time and the largest longitudinal telomere study outside the human literature so far. Our results suggest that individual cattle differ in their RLTL dynamics over life. Although most of the difference between animals is explained by a different average RLTL (intercept) (94.7%), a small proportion is due to different shapes of RLTL profiles (5.3 %). This is an important observation that justifies the further investigation of differences in telomere profiles in association with traits of interest such as health, fertility and mortality. The only other study we are aware of that used random regression models for the analysis of longitudinal telomere data did not report a significant difference in telomere dynamics among Seychelles warblers (Barrett et al. 2013), which might have been due to the relatively small sample size of that study.

At a population level RLTL shortened in the beginning of life. The fixed curve calculated in the present study suggests an average RLTL increase later in life. However, this is probably due to the symmetry of a quadratic function and might not reflect biological changes. Therefore, we argue that at a population level telomeres shorten in the beginning of life and remain relatively stable thereafter. Some previous longitudinal studies in baboons and birds support these results, though they did not use random regression models for their analyses (Baerlocher et al. 2007; Hall et al. 2004). A study in humans found that the early life telomere attrition was followed by a plateau with no telomere change and by a second decline in telomere length as adults grew older (Frenck et al. 1998)(Frenck et al. 1998). It is possible that our study did not include animals that were old enough to show that second decline.

In the present study we report the first heritability estimates for telomere length across all species that were calculated using random regression models. Random regression model estimates do not only inform about the proportion of the variance that is due to additive genetic effects, they also demonstrate how this proportion might change over time. It is known that telomere length is affected by many different genes (Pooley et al. 2013; Gatbonton et al. 2006; Levy et al. 2010; Shen et al. 2011; Mangino et al. 2008). Epigenetic changes to the genome can alter the translation of genes with ageing (Issa 2003; Pal & Tyler 2016). If regulatory genes for RLTL were activated or silenced in an unbalanced manner with ageing,

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heritability estimates for RLTL might change considerably. However, in the present study we show that heritability estimates for bovine RLTL are not only relatively high (0.36 to 0.47; SE= 0.05-0.10) they are also relatively stable (Fig 3). This means that RLTL at all ages could be influenced by breeding programmes. Heritability of telomere length estimated with relatively simpler models has been reported before in humans (0.39- 0.82) (Vasa-Nicotera et al. 2005; Bischoff et al. 2005; Broer et al. 2013; Hjelmborg et al. 2015; Njajou et al. 2007), sand lizards (0.52) (Olsson, Pauliny, Wapstra, Uller, Schwartz & Blomqvist 2011) and kakapos (0.42-0.77)(Horn et al. 2011).

Within an animal, the genetic correlation between consecutive RLTL measurements decreased as the time interval between measurements increased. This suggests that RLTL might be under different genetic control at different life stages. As mentioned before, epigenetic changes during ageing (Pal & Tyler 2016; Issa 2003) might inhibit or promote genes that play a role in telomere maintenance. Also, telomeres have been reported to have regulatory functions themselves that act on genes in their close proximity and even in further distance (Baur et al. 2001; Robin et al. 2014; Kim et al. 2016). For example, long telomeres form bulky structures that can inhibit transcription of genes in their neighbourhood. When telomeres shorten they unfold and enable the expression of those genes. This is known as telomere positioning effect (Baur et al. 2001). Also, shelterin proteins can act as transcription factors and thus regulate gene expression (Ye et al. 2014).

Not much is known about telomere length and its association with productive lifespan in cattle so far. In cross-sectional studies bovine telomere length declines with age and during the lactation period (Miyashita et al. 2002; Brown et al. 2012; Laubenthal et al. 2016). A single study found that animals with shorter telomeres were more likely to be culled within the next year (Brown et al. 2012). In the present study we did not find a significant relationship between telomere dynamics and productive lifespan in cattle. Dairy cattle rarely live until their physiological end of life but are usually culled for fertility, productivity or health reasons. In the introduction we argued that productive lifespan was still biologically meaningful, because animals are not randomly selected for culling. However, the relationship between productive lifespan and RLTL might be different than these relationships in humans or natural animal populations. Also, a relationship between RLTL and productive lifespan in dairy cattle might be there if RLTL change was examined in a different

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way. RLTL dynamics might be too pulsatile to be exactly described by random regression models. Future studies are required to investigate the best way to analyse longitudinal datasets that include more than two RLTL measurements per animal. While current results did not show a significant correlation between RLTL and productive life at phenotypic level, a further study examining genetic correlation between the two traits is of high interest as it may provide a different result.

4.7. Thesis context

The present chapter contributes towards the third and fourth objectives of this thesis. RLTL dynamics were investigated at the population level and at the individual animal level. Also, genetic parameters of RLTL were calculated and the association of different RLTL dynamics with productive lifespan was investigated. While Chapter 3 showed that RLTL at population level declines early in life and remains relatively stable thereafter, this chapter shows that individual animals differ significantly in their RLTL changing patterns. Furthermore, the present chapter deepens the heritability analysis of Chapter 3. While in the previous chapter an overall heritability estimate was calculated, the change of heritability with age was illustrated in the present chapter, showing that heritability remained relatively stable over the productive lifespan of the dairy cattle in this study. Also, the present chapter adds to the genetic literature on telomere length by analysing the within trait correlation over time and showing that RLTL in early life might be partially under different genetic control than RLTL later in life. Finally, the association between individual animal RLTL changing profile over life and productive life was analysed but no significant relationship was found. This is further addressed in the following chapter of the thesis where change in telomere length is investigated in a different way, because quadratic curves (as used in the present chapter) might be too restrictive to capture the complexity of more pulsatile telomere change.

4.8. Chapter appendix (Supplementary File 1)

4.8.1. Model building

We evolved the mixed models used in Chapter 3 into random regression models. The initial model used in the previous study included the animal identity as a random effect and the following fixed effects: age group of the animal (younger or older than two months) birth year, qPCR plate on which the particular sample was measured, and the qPCR row. For the genetic analysis of RLTL in the present study we also added the genetic group of the animal as an additional fixed effect. For random regression analysis, the time measurements (age) were transformed to a continuous scale (as age in months) to facilitate the interpretation of results.

The significance of the animal identity as random effect was tested by comparing Akaike information criterion (AIC) of a model that only included fixed effects with the model that also included animal identity as a random effect. The delta AIC was 204.97 indicating that the random effect is statistically highly significant (Table S1). This implies that animals differ in their intercepts and thus in their average RLTL over life. Next, we added pedigree information to the animal identity and compared AIC values with the former model. The pedigree information improved the fit further (delta AIC= 55.46) (Table S1). We fitted the permanent environment as an additional random effect which seemed to have a marginally significant effect on the model fit (delta AIC = 2) (Table S1). However, the effect size of the permanent environment effect was marginal with a relatively large standard error (permanent environment effect variance =0.2 E-09, SE - standard error not estimable, compared to an additive genetic variance= 0.1 E-02, SE =1.9 E -04). We fitted the permanent environment effect to the final random regression model which caused a failure of convergence. Because of the minimal effect size of the permanent environment effect it was judged to be negligible in the present study and therefore removed from further analyses.

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Table S1: Comparison of models including different random effects. Fixed effects in the model were: Age in months, genetic group, birth year, qPCR plate and qPCR row. AIC – Akaike information criterion, logL – log-likelihood at convergence, logLratio – twice the difference in logL between the models.

Model	random effect	AIC	compared with	delta AIC	LogL	logLratio
Model 0	/	-5639.91	model 1	204.97	2820.96	206.96
model 1	animal identity	-5844.88	model 2	55.46	2924.44	55.46
model 2	additive genetic effect	-5900.34	model 2	0	2952.17	0
model 3	additive genetic effect + permanent environment	-5898.34	model 2	2	2952.17	0

Next, we added orthogonal Legendre polynomials of increasing order to the fixed effect of age in months and determined the best fitting order by comparing AIC values. A cubic function of age fitted the data best (Table S2). However, when the same order of Legendre polynomial was fitted to the random effect of the animal identity, the model failed to converge. Therefore, the model was simplified by using a quadratic polynomial for both the fixed and the random effect which allowed convergence.

Table S2: Comparison of models including different orders of Legendre polynomials fitted to the fixed effect of age in months. Other fixed effects in the model were: genetic group, birth year, qPCR plate and qPCR row while animal identity with associated pedigree information (additive genetic effect) was fitted as a random effect

Model	Order of Legendre Polynomial	AIC	delta AIC
model 1	NULL	-5900.34	74.66
model 2	linear	-5907.18	67.82
model 3	quadratic	-5963.41	11.59
model 4	cubic	-5975	0
model 5	quartic	-5970.12	4.88

The inclusion of the quadratic polynomial to the animal identity (random effect) was statistically significant (delta AIC = 3.24). This implies that animals differ in their telomere length profiles and thus in their direction and amount of RLTL change. The quadratic polynomial also described individual deviations from the fixed curve better than a linear function (delta AIC = 2.04) (Table S3).

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Table S3: Comparison of different Legendre polynomials fitted to the additive genetic effect of the animal identity. Other fixed effects in the model were age in months (with fitted polynomial), genetic group, birth year, qPCR plate and qPCR row.

Model	Legendre polynomial (fixed)	Legendre polynomial (random)	AIC	compared to	delta AIC	LogL	logL-ratio
model 8	cubic	cubic	not converged	/	/	/	/
model 9	quadratic	quadratic	-5966.65	model 9	0	2990.32	0
model 10	quadratic	linear	-5964.58	model 9	2.07	2986.29	8.06
model 11	quadratic	NULL	-5963.41	model 9	3.24	2983.7	13.24

Next, we tested in the random regression model if a heterogeneous variance structure improved the model fit further. First we considered four and then two different variance groups (Table S4).

Table S4: Testing a heterogeneous variance for 1) four different age groups 2) two different age groups; σ^2 – the residual variance estimate; SE – standard error

	age group	σ^2	σ^2/SE	SE	N
1)	0-12 months	2.57E-03	11.2	2.29E-04	465
	13-24 months	1.96E-03	8.55	2.30E-04	255
	25- 40 months	2.35E-03	10.6	2.22E-04	368
	> 40 months	2.05E-03	7.86	2.61E-04	240
2)	< 2 months	2.37E-03	8.17	2.90E-04	310
	> 2 months	2.27E-03	17.9	1.27E-04	1018

Differences in the residual variance between considered age groups were not statistically significant ($p=0.246$ and $p= 0.721$ for four and two age groups, respectively). Models with homogeneous and heterogeneous variance structures were also compared based on their AIC values. The difference between a homogeneous variance structure and two variance groups was not statistically significant (delta AIC = 1.89), while the homogeneous variance structure was a better fit than four variance groups (delta AIC= 2.09). Therefore, a homogeneous variance structure was assumed in all following models.

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4.8.2. Calculation of genetic parameters

The phenotypic variance (V_P) for each month equalled $V_A + V_E$, where V_E = environmental variance and V_A =additive genetic variance. The heritability (h^2) was calculated for each month as follows (Falconer & Mackay 1996):

$$h^2 = \frac{V_A}{V_P} \quad (\text{S } 1)$$

The standard error (SE) of h^2 was calculated using following formula (Gilmour et al. 2009):

$$SE = \sqrt{(h^2)^2 * \left(\frac{\text{var}(V_A)}{V_A^2} + \frac{\text{var}(V_P)}{V_P^2} - 2 * \frac{\text{cov}(V_P, V_A)}{V_A V_P} \right)} \quad (\text{S } 2)$$

Genetic covariances between different ages in months were calculated using equation (3) in the manuscript except that φ' denoted the transpose vector including the residuals for each polynomial order for a different month than φ . The genetic correlations between RLTL in different months were then calculated using following formula (Falconer & Mackay 1996):

$$r_A = \frac{\text{cov}_{xy}}{\sqrt{\text{var}_x \text{var}_y}} \quad (\text{S } 3)$$

where cov_{xy} was the genetic covariance between months x and y and var_x and var_y were the corresponding genetic variance estimates.

Eigenfunctions were calculated by multiplying a matrix containing eigenvectors with a matrix containing Legendre polynomial residuals for each order of the polynomial function and each month. Following formula was used:

$$\psi_i(x) = \sum_{j=0}^{p-1} [k_{\psi_i}]_j \Phi_j(x) \quad (\text{S4})$$

Where $[k_{\psi_i}]$ is j^{th} element of the i^{th} eigenvector K, Φ is the j^{th} polynomial of the order p and x is the age in months.

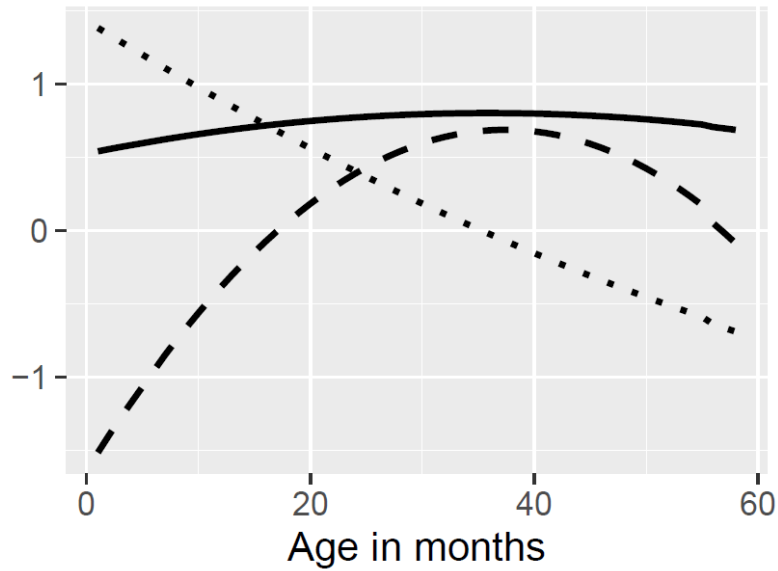


Figure S1: Eigenfunctions (y-axis, unitless) over age in months associated with the largest (intercept; solid line), middle (linear; dotted line) and smallest (quadratic; dashed line) eigenvalue.

4.8.3. Output of the final random regression model

Table S5: Significance of fixed effects in the final random regression model. A quadratic Legendre polynomial was fitted to age in months. * Significant at $p=0.05$, ** significant at $p = 0.01$, *** significant at $p= 0.001$.

Source of variation	F statistic	p-value	
Intercept	66.01	0.001	**
Genetic group	0.02	0.871	
age in months	16.99	0.023	*
Birth year	1.76	0.107	
qPCR row	34.53	<0.001	***
qPCR plate	5.98	<0.001	***

Chapter 5:

The amount of telomere attrition is associated with productive lifespan in dairy cattle

5.1. Abstract

Telomeres are the ends of eukaryote linear chromosomes. They have been shown to shorten with age in many species. In humans lifestyle factors such as smoking, exercise and stress were linked to telomere shortening and short telomeres were associated with a range of diseases such as diabetes mellitus, mild cognitive impairment and cardiovascular disease. Also, telomere length may be associated with lifespan in different species. Factors that influence telomere change in dairy cattle are largely unknown and also the association of telomere length and telomere length dynamics with the productive lifespan (time from birth to culling) of dairy cows requires further exploration. The present longitudinal study is based on 1328 relative leukocyte telomere length (RLTL) measurements of 308 animals. Factors that influenced the amount and direction of RLTL change were investigated using a mixed model. Age at sampling significantly affected RLTL change ($p < 0.001$). Telomere attrition was more prominent early in life than at older ages. Autocorrelations between consecutive RLTL measurements were overall moderate and significant ($r = 0.38$ $p < 0.001$). The effect of three different measures of lifetime RLTL dynamics on productive lifespan was investigated: 1) mean RLTL over life, 2) mean RLTL change and 3) mean absolute RLTL change. Mean RLTL change was significantly associated with productive lifespan. Animals with more telomere attrition on average were culled at a younger age ($p < 0.001$). The relationship remained statistically significant when animals with less than three samples were excluded from the dataset ($p = 0.047$). A possible application of monitoring RLTL change in dairy cattle may be the use as a biomarker for animal welfare, but future studies have to investigate the relationship between telomere change and influencing factors in more detail.

5.2. Introduction

Telomeres are structures at the ends of linear chromosomes that consist of repetitive DNA nucleotides and proteins (Blackburn & Gall 1978; De Lange 2005).

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Telomere length (TL) differs between tissues, cells and chromosome ends (Friedrich et al., 2000; Lin et al., 2010, 2016; Martens et al., 1998; Nasir et al., 2001) and is negatively correlated with the number of cell divisions (Harley et al., 1990; Olovnikov, 1973; Watson, 1972) and the exposure to oxidative stress (von Zglinicki 2002). Both long and short telomeres have been shown to affect the functionality of the cell. Critically short telomeres trigger a DNA damage response that leads to replicative senescence or apoptosis (Fagagna et al. 2003; Harley, Futcher & CW. Greider 1990; Bodnar et al. 1998). Long telomeres on the other hand were shown to be more susceptible to gamma irradiation leading to a decreased survival of cancer cells with extremely long telomeres (Fairlie & Harrington 2015). Telomere length at a cellular level is tightly regulated. Short telomeres can be replenished by expression of the reverse transcriptase telomerase (Blackburn et al., 2006; Greider & Blackburn, 1985) or by the “alternative lengthening of telomeres” (ALT) (Cesare & Reddel, 2008, 2010; Henson et al., 2002; Londoño-Vallejo et al., 2004; Neumann et al., 2013), whereas long telomeres can be shortened by telomere trimming (Li et al., 2017; Pickett et al., 2009; Pickett et al., 2011; Pickett & Reddel, 2012).

In humans, leukocyte telomere length seems to change with age following a general pattern with three different phases: 1) Fast TL attrition in early life, 2) a plateau with little TL change, 3) a second phase of slower TL attrition later in life (Aubert & Lansdorp, 2008; Frenck et al., 1998). At all ages the variance of TL within an age group is typically large. Both genetic (Pooley et al. 2013; Aviv 2012; Asghar et al. 2015) as well as lifestyle factors such as smoking (Huzen et al. 2014; Mirabello et al. 2009), body mass index (Kim et al. 2009), physical activity (Cherkas et al. 2008), relaxation techniques (Hoge et al. 2013), and psychological stress (Epel et al. 2004) were shown to impact TL. Both extremely long and extremely short telomeres have been associated with diseases and higher mortality in humans (Stanley & Armanios 2015). Relatively short telomeres in relation to age group peers were linked to an early onset of age related symptoms and diseases such as type 2 diabetes mellitus (Salpea et al., 2010; Zhao et al., 2013), cardiovascular disease (Yeh & Wang 2016; Haycock et al. 2014; Aviv 2012), cognitive impairment (Hägg et al., 2017; Hochstrasser et al., 2011), brain atrophy (Wikgren et al. 2014) and overall cancer incidence (Willeit et. al, 2011). Also, shorter telomeres were shown to be associated with a shorter subsequent lifespan (Bakaysa et al., 2007; Cawthon et al., 2003). Long telomeres on the other hand were correlated with a higher risk of specific

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cancer types such as melanomas (Stanley & Armanios 2015). However, other studies did not find a relationship between telomere length and diseases such as type 2 diabetes mellitus (Hovatta et al. 2012) or cardiovascular disease (Raymond et al., 2012) or mortality (Martin-Ruiz et al., 2005). It is unclear why studies report such mixed results.

Most studies on TL in relation with diseases and longevity were based on just a single or two TL measurements per individual. Longitudinal studies with a larger number of repeat samples per individual are largely missing from the literature. Such detailed longitudinal studies are urgently required to better understand the degree to which within individual telomere dynamics throughout life versus constitutive among individual differences in TL drive associations between TL, health and longevity (Boonekamp et al., 2013; Hornsby, 2006; Lansdorp, 2006; Mather et al., 2011).

In non-human vertebrates, a recent meta-analysis demonstrated that generally shorter blood cell TL is associated with increased subsequent mortality risk across species (Wilbourn et al. 2018). However, a variable pattern of blood cell TL change with age has been observed across species. In most endothermic vertebrate species studied to date, rapid shortening of TL is observed during early life, as in humans (Fairlie et al. 2015; Baerlocher et al. 2007; Heidinger et al. 2012, Chapters 3 and 4 of this thesis). Whilst a secondary decline in TL with age during adult life has been observed in many species such as cats, dogs, sheep, badgers and goats (Brümmendorf et al. 2002; Benetos et al. 2011; Alexander et al. 2007; Betts et al. 2005; Beirne et al. 2014), little or no change with age in adulthood has been observed in other mammal and bird species (Baerlocher et al. 2007; Hall et al. 2004; Fairlie et al. 2015, Chapters 3 and 4 of this thesis). Recently, longitudinal studies have also revealed evidence of within-individual increases in TL with age during certain life stages in endothermic vertebrates (Hoelzl et al. 2016; Cerchiara et al. 2017). Complex telomere dynamics across age have also been observed in ectothermic vertebrates; for example in both wild tropical pythons and captive zebra fish erythrocyte TL shortens during early life, then elongates and shortens thereafter again (Ujvari & Madsen 2009; Anchelin et al. 2011). Currently, the evolutionary and ecological factors responsible for these among species differences and the relationship between patterns of TL change with age and survival or fitness are largely unknown.

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Two distinct but mutually non-exclusive ideas regarding the importance of among- and within-individual variation in TL have emerged recently from longitudinal studies of vertebrate blood cell TL. A human study, which measured leukocyte TL twice in the same individuals from four different study cohorts spanning ages at baseline from 30 to 75 years and an average sampling interval of 12 years, found that the rank order of TL among individuals is largely maintained across these timescales (Benetos et al. 2013). They argued that genetic and early-life environmental determinants of TL lead to strong and persistent among-individual differences in TL throughout adult life (Benetos et al. 2013). At the same time, evidence is mounting from both human and other vertebrate studies that blood cell TL is a dynamic measure, and many studies showed TL shortening in response to various lifestyle factors in humans (Huzen et al. 2014; Mirabello et al. 2009; Kim et al. 2009; Cherkas et al. 2008; Hoge et al. 2013; Epel et al. 2009) and to both environmental conditions and experimental induction of stress in birds (Angelier et al., 2013; Asghar et al., 2015; Bauch et al., 2013; Haussmann et al., 2012; Monaghan, 2014; Nettle et al., 2013; Reichert et al., 2014). A growing number of longitudinal follow-up studies in such systems, typically across just a small proportion of the organism's life expectancy, suggest wide variation in both the magnitude and direction of TL change over time (Berglund et al., 2016; Bize et al., 2009; Boonekamp et al., 2014; Hall et al., 2004; Hovatta et al., 2012; Steenstrup et al., 2013). In most of those studies a subset of individuals even lengthened their telomeres over time (Aviv et al., 2009; Bize et al., 2009; Chen et al., 2011; Ehrlenbach et al., 2009; Fairlie et al., 2015; Hoelzl et al., 2016; Nordfjäll et al., 2009). In light of the large variation in telomere change, it has been proposed that blood cell TL may represent a marker of the 'exposome': the total exposure to environmental and physiological stressors over the life course (Blackburn et al., 2015). Studies showing high individual repeatability of TL over time argue that it is early life TL or mean lifetime TL that is likely to be important for predicting health outcomes (Benetos et al. 2013; Heidinger et al. 2012). However, studies pointing to highly dynamic TL fluctuations over the lifetime suggest that TL shortening in response to the accumulation of environmental stress exposure is likely to be the best predictor of health (Blackburn et al., 2015; Epel et al., 2004; Herborn et al., 2014; Nettle et al., 2013).

Several longitudinal studies indicate that the amount and direction of within individual telomere change is biologically meaningful. In birds (Alpine swifts,

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Seychelles warblers and jackdaws) faster erythrocyte telomere attrition was associated with poorer survival (Barrett et.al, 2013; Bize et al., 2009; Boonekamp et al., 2014). A study in humans found that not only the amount of change but also the direction of TL change was associated with survival: telomere maintenance or elongation was beneficial compared to telomere attrition in patients with cardiovascular disease (Goglin et al. 2016). However, telomere change has not been analysed in relation with health and longevity in studies that included repeated TL measurements over the whole lifetime of the study species. If more than two samples are present for each individual the question of telomere change extends from a scenario where telomeres can be maintained, shortened or elongated to the inclusion of complex within individual patterns that allow oscillation of TL measurements over life.

If we accept that within-individual changes in TL reflect biological processes and not measurement error – as has recently been argued (Bateson & Nettle 2016) – then a further important question is of the degree to which variation in the amount and direction of change reflects simply variation in exposure to environmental stressors or the ability of the individual to buffer itself physiologically from that exposure. Under the former, the amount and direction of telomere change would depend on the accumulation and intensity of stressors. Under the latter, animals would differ in their amount (and direction) of telomere change even when challenged with the same kind and intensity of stressor. In wild populations and humans it is difficult to differentiate between those two scenarios because individuals live under different conditions and face a variety of stressors. The opposite is true for livestock.

In the present study we investigate the change of relative leukocyte TL (RLTL) in a population of Holstein Friesian dairy cattle that is kept on a single farm where all animals are naturally challenged with similar stressors. Even though most dairy cattle do not reach their physiological end of life, the productive lifespan (time from birth to culling, i.e. removal from the herd) is informative about biological processes, because all animals are culled for reasons related to productivity, poor health or fertility. Although animals in the present study live under a controlled environment, they still differ in the amount and severity of diseases.

In the previous two chapters of this thesis it was shown that bovine RLTL remained stable after the initial decline in early life at a population level. Telomere dynamics within individual cattle has not been studied before, except in the previous chapter of

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this thesis where random regression animal models were used to reveal that animals differed significantly in their average RLTL (intercept) and their changing pattern over life (slope) (Seeker et al. 2018). Although random regression models were helpful to understand the change of genetic parameters such as heritability over life, they might have been too restrictive to sufficiently explore the change of RLTL within the individual. Random regression models are usually applied to traits that change relatively slowly with time such as body condition score (Banos et al., 2004; Oikonomou et al., 2008) or milk yield (Mrode & Coffey 2008) and do not fluctuate rapidly. Figure 1 illustrates the limitation by showing predicted individual age slopes from our random regression models for RLTL for all animals with at least seven measurements. The predicted slopes are shown in relation to the animals' RLTL measurements that were adjusted for qPCR plate and row. It can be seen that random curves are relatively similar and show only little RLTL change within individual. However, telomere residuals deviate considerably from those curves. For the purposes of the previous chapter it was assumed that this deviation was due to measurement error. However, the deviation might also reflect biological processes that are too complex to be captured by a quadratic polynomial function that are worth investigating. Another feature of random regression models is that they predict measurements for missing measurement times for all animals in the study. This goes so far that RLTL is predicted for animals even after their death. Predicting missing values is likely to be more accurate for traits that change slowly and predominantly in one direction but not reliable for traits that might change rapidly. Also, predicting measurements after the death of the animals does not make biological sense and may influence the analysis in an unpredictable way. For all those reasons, in the present chapter change is calculated as the difference between two consecutive RLTL measurements that were pre-adjusted for qPCR plate and row without the use of polynomials.

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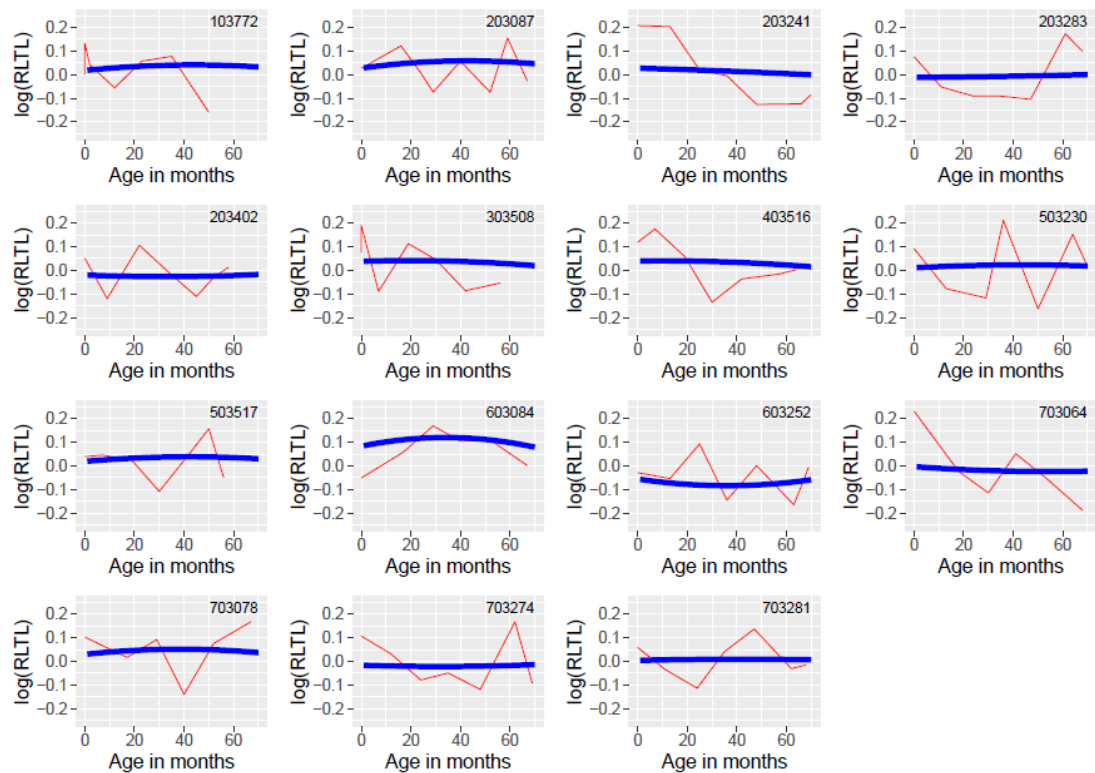


Figure 1: Individual profiles of 15 different animals. Random curve calculated with random regression models is shown in blue. In red are RLTL measures corrected for qPCR plate and row; source: Chapter 4 of this thesis.

The objectives of the present chapter are to 1) test if dairy cattle differ in their direction and amount of RLTL change across measurements, 2) investigate which factors influence the amount and/or direction of RLTL change, 3) analyse the relationship between average RLTL over life with productive life span, and 4) investigate the association of the amount and direction of RLTL change over life with productive lifespan.

5.3. Materials and Methods

5.3.1. Animal population and data collection

Female Holstein Friesian dairy cattle of the Langhill herd were kept at the Crichton Royal Research Farm in Dumfries (Scotland). At the farm cows of two distinct genetic lines (a select line for high milk fat and protein yield and a control line) are randomly allocated to two different diets. One diet consists of a higher proportion of forage and therefore contains less energy, whereas the other is richer in energy (Veerkamp et al. 1994). The Langhill herd is intensely monitored for a broad range

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of measurements, such as body weight, feed intake, signs of disease (health events), milk yield, productive lifespan and reasons for culling.

5.3.2. Blood sampling, DNA extraction and qPCR

For the present study we selected animals that were born between 2008 and 2014 and were equally distributed across both genetic lines and feeding groups. Whole blood samples were taken by venepuncture within 15 days after birth and at least one more time later in life. On average 4.3 samples (range: 2-8) were taken for each animal. Whole blood samples were stored with EDTA as anticoagulant at -30 ° C until DNA extraction with the DNeasy Blood and Tissue spin column kit (QIAGEN). DNA samples had to have a minimum yield, purity and integrity to pass our internal quality control. Yield and purity were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and the DNA integrity was evaluated on integrity gels following Seeker et al. (2016). In total, 1,328 samples of 308 animals with a minimal concentration of 20 ng/μl and ratios of 260/280 > 1.7 and 260/230 >1.8 that also had an DNA integrity score of 1 or 2 (Seeker et al. 2016) were included on qPCR plates for RLTL measurements. We measured telomeric DNA in relation to the reference gene beta-2-microglobulin (B2M) that is constant in copy number (Seeker et al. 2016). Both reactions were performed on the same qPCR plate but in different wells (monoplex qPCR). An identical sample was included as a calibrator (or “golden sample”) twice on all 25 qPCR plates: one time in the middle of the plate and another time at its periphery. The measurements for the calibrator sample were used in the calculation of RLTL of individual samples to correct for part of the random measurement error that was associated with the qPCR plate. The two locations of the calibrator were used to test for a qPCR plate edge effect. All other samples were randomly allocated to qPCR plates and wells. Also, a negative control (DNAse and RNase free water) and a serial dilution of the calibrator DNA for visual qPCR quality control were added to each qPCR plate. A liquid handling robot (Freedom Evo by TECAN) was used to load samples, the calibrator, the negative control and the serial dilution in triplicates onto 384 well qPCR plates. For the amplification of telomeres tel 1b (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT) and tel 2b (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT) primers were used (Epel et al. 2004). B2M primers were obtained from (Primerdesign, accession code NM_001009284).

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The software LinReg PCR (Ruijter et al. 2009) was used for fluorescence baseline correction of raw RLTL measurements and for the calculation of reaction specific qPCR efficiencies for each plate (E_{TEL} and E_{B2M} for the telomere and the B2M reaction respectively). The following formula was used for RLTL calculation (Pfaffl 2001):

$$RLTL = \frac{E_{TEL}^{Cq_{TEL(Calibrator)} - Cq_{TEL(Sample)}}}{E_{B2M}^{Cq_{B2M(Calibrator)} - Cq_{B2M(Sample)}}} \quad (1)$$

The Cq value describes the number of cycles of a qPCR that is required for an amplification curve to cross a set fluorescence threshold. The Cq values of the calibrator sample were $Cq_{TEL(Calibrator)}$ and $Cq_{B2M(Calibrator)}$ for the telomere and the B2M reaction respectively. Cq values of the individual samples were $Cq_{TEL(Sample)}$ and $Cq_{B2M(Sample)}$.

5.3.3. Statistical analysis

In total, 1,328 samples of 308 animals passed all DNA quality control steps and were used for RLTL measurements. Table 1 summarises how many animals with a specific number of RLTL measurements were available. RLTL data were pre-adjusted for qPCR plate and row by fitting both as fixed effects in a linear model prior to any calculations, in order to remove technical variation identified in chapters 3 and 4.

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Table 1: Number of animals with a specific number of RLTL measurements (1-8). Only DNA that passed all quality control steps was used for RLTL measurement. Three animals were left with a single sample after quality control. All remaining animals had longitudinal samples.

Total number of animals	Total number of RLTL measurements	Number of RLTL measurements per animal							
		1	2	3	4	5	6	7	8
308	1,328	3	28	38	106	83	35	14	1

For each individual, we calculated the change in adjusted RLTL as the difference between consecutive measurements for all pairs of measures available (“RLTL change” = RLTL at time t minus RLTL at time t-1). The percentage of occasions in which animals shortened or elongated their telomeres between consecutive measurements was calculated. The absolute magnitude of change between shortening and lengthening occasions was compared using a Welch’s t-test.

To investigate which factors affect the direction and amount of RLTL change a linear mixed model was fitted with RLTL change as response variable and animal identity as random effect. The following factors were included as fixed effects in the model to test their impact on RLTL change: Genetic line, feed group and birth year of the animal, age at sampling (at time t), and the occurrence of a health event within two weeks before or after sampling (at time t). The time difference between consecutive samplings in days was fitted as a covariate. Non-significant effects ($p > 0.05$) were backwards eliminated from the model. Age at sampling was modelled as a covariate (age in months or years) and as a two level factor (sampling at ages younger and older than one year); the best fitting age term was selected by comparing the Akaike information criteria (AIC) of different models where all other factors remained the same. The most parsimonious model including the best fitting age term was used to calculate the repeatability of RLTL change as the fraction of the variance that is due to the animal divided by the total phenotypic variance. To test if the magnitude of RLTL change (regardless of the direction) is repeatable, the model was re-run with absolute RLTL change as response variable.

To better understand the dynamics of RLTL length within the individual, correlations between all consecutive RLTL measurements were calculated and plotted. Additionally, a mixed model with RLTL at time t as response variable, animal identity as random effect and RLTL at time t-1 was run to test the degree to which RLTL measurements depended on the previous RLTL measurements. The following

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additional fixed effects were tested in this model and backwards eliminated if not significant: genetic group, feed group and birth year of the animal, age at sampling, the time difference between sampling in days, and the occurrence of a health event within two weeks of sampling.

Next, three measures of lifetime within-individual telomere dynamics for all 244 dead individuals in the present study were calculated: the mean residual RLTL of each individual as a measure of average RLTL across life (“mean RLTL”), the mean of all RLTL change values for each individual (“mean RLTL change”) and the mean of all absolute RLTL change values for each individual (“mean absolute RLTL change”). Mean RLTL change captures both the direction and magnitude of RLTL change over an individual’s lifetime whilst mean absolute RLTL change measures only the magnitude of change irrespective of direction. Correlations between all three measures of lifetime RLTL dynamics were calculated and plotted to ensure that they were not too similar but represented different aspects of RLTL dynamics.

We were also interested to test if the three different measures of lifetime RLTL dynamics were associated with the productive lifespan of the animals. We define “productive lifespan” as the time between birth and culling in days. Out of 308 animals, 244 had died and therefore provided an accurate and unbiased measure for their productive lifespan. For those 244 animals and their 1,015 samples the effects of 1) mean RLTL 2) mean RLTL change and 3) mean absolute RLTL change on productive lifespan were tested using general linear models. By comparing the fit of all three metrics in our models of productive lifespan we aimed to test whether relationships were driven specifically by mean RLTL, telomere attrition or gain or by differences in the amplitude of oscillations in telomere length. First separate models were used for each measure of lifetime RLTL dynamics to test their effect on productive lifespan individually. Because the effect of different lifetime RLTL dynamics measurements might partly be overlapping, all three measures were included in the same model to test which effect remained significant while accounting for the two other lifetime RLTL dynamics measures.

In the previous two chapters of this thesis it was reported that RLTL shortens significantly within the first year of life and remains relatively stable afterwards at a population level. Therefore, an association between telomere attrition and productive lifespan might be confounded with the number of available samples: If animals shortened their telomeres within the first few months of life and died

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thereafter, a dramatic RLTL change and a short lifespan would probably correlate without having a causal relationship. To test this possibility the analysis of productive lifespan in association with mean RLTL change was repeated for all animals with a minimum of three RLTL measurements (212 animals).

All statistical analyses were performed in R studio with R 3.1.3. (R Core Team 2014). Mixed-effects models were implemented using the 'lme4' library.

5.4. Results

Animals varied in the amount and direction of RLTL change. Adjusted RLTL and RLTL change were approximately normally distributed (Fig. 2). Based on 1020 possible pairwise RLTL change measurements (Table 1), animals shortened their telomeres in 56.76 % of all occasions and lengthened them in 43.24%. There was a statistically significant difference between the magnitude of change depending on the direction of change: shortening events dominated over elongation events (mean shortening: 0.154, mean elongation 0.127, $p < 0.001$).

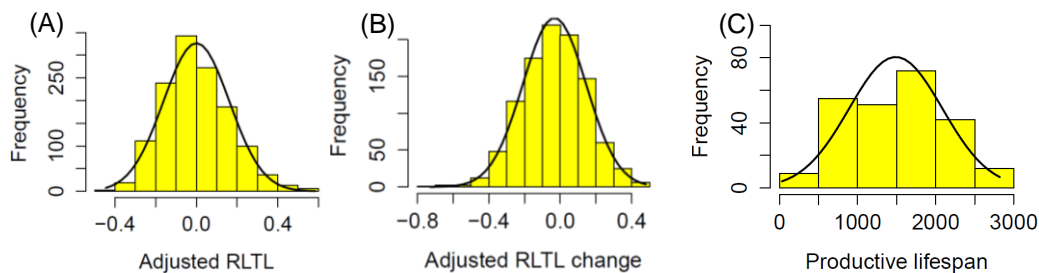


Figure 2: Histograms of (A) RLTL residuals, (B) RLTL change and (C) productive lifespan.

Only age at sampling had a significant effect on RLTL change. Genetic group, feed group and birth year of the animal, the time difference between sampling in days, and the occurrence of a health event within two weeks of sampling had no statistically significant effect on RLTL change ($p > 0.05$) and therefore were removed from the model. The best fitting age term was age in years (AIC= -664.63 compared to age in months: AIC= -659.13 and age as a two level factor: AIC= -649.99). Age at sampling significantly affected RLTL change (Estimate: 0.03, $p < 0.001$). Telomere attrition was more prominent at younger ages than at older ages (Figure 3 A). RLTL change was not significantly repeatable within individuals (repeatability = 0). However, consecutive RLTL measurements within the individual were overall moderately positively and significantly correlated ($r = 0.38$, $p < 0.001$; Figure 3 B). This

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result supports the already established moderate and significant individual repeatability of RLTL in our study system presented in Chapters 3 and 4. Tested with a mixed model, RLTL at time t was more affected by RLTL at time $t-1$ (estimate=0.17, $p<0.001$; Figure 3) than by age in years (estimate= 0.008, $p=0.02$).

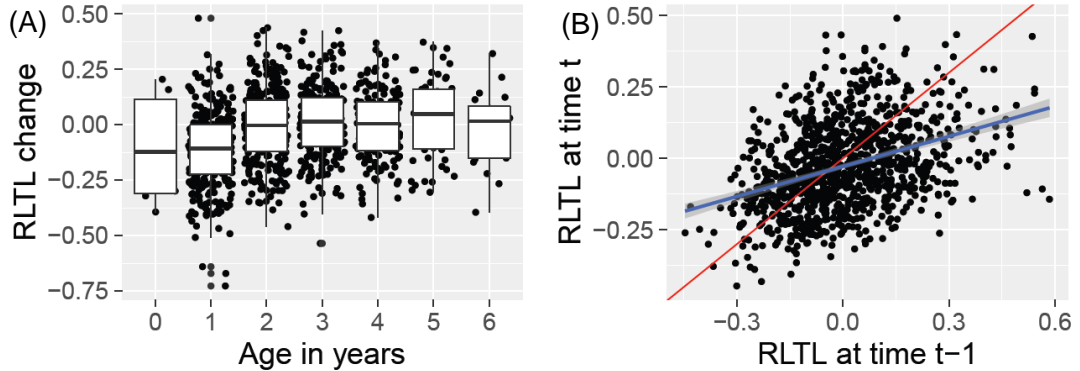


Figure 3: Factors that influence RLTL change. (A) Age in years is significantly associated with the amount and direction of RLTL change (Estimate: 0.03, $p<0.001$). **(B)** At all measurement times the present measurement (RLTL at time t) is clearly affected by the previous measurement (RLTL at time $t-1$) (estimate=0.17, $p<0.001$). The red line represents a perfect correlation.

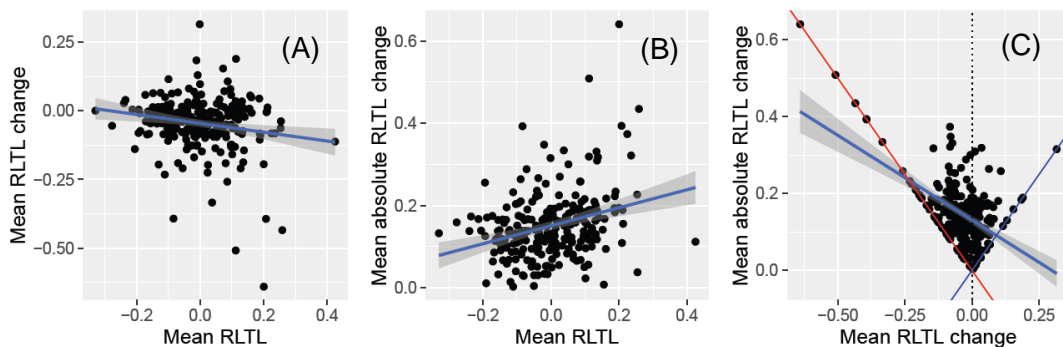


Figure 4: Correlations between three different measures of lifetime relative leukocyte telomere length (RLTL) dynamics calculated for 244 dead animals. (A) Mean RLTL and mean RLTL change: $r=-0.18$, $p=0.004$; **(B)** Mean RLTL and mean absolute RLTL change: $r=0.30$, $p<0.001$. **(C)** Mean RLTL change and Mean absolute RLTL change: $r=-0.53$, $p<0.001$. The red line represents perfect negative correlation, blue line represents perfect positive correlation.

Next, three different measures for lifetime RLTL dynamics were calculated and the correlations between all those measures are visualised in Figure 4. Mean RLTL and mean RLTL change were significantly negatively correlated ($r=-0.18$, $p=0.004$). Animals with on average long telomeres were more likely to shorten their telomeres

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(Figure 4 A). Also, the absolute amount of telomere change was positively correlated with mean RLTL length ($r=0.30$, $p<0.001$) showing that animals with long telomeres not only showed more attrition but more RLTL change in general (Figure 4 B). Mean RLTL change and mean absolute RLTL change were moderately but significantly negatively correlated ($r= -0.53$, $p<0.001$, Fig. 4 C) illustrating that they represent related but different measures of RLTL dynamics.

The main purpose of the calculation of lifetime RLTL dynamics measured was to investigate if there was a relationship between mean RLTL or RLTL change (both considering and disregarding the direction of change) with productive lifespan. When each measure of lifetime RLTL dynamics was tested in a separate general linear model, mean RLTL did not affect productive lifespan significantly ($p=0.44$; Figure 5 A). However, mean RLTL change (Estimate= 1685.07, SE= 368.30, $p<0.001$) and mean absolute RLTL change (Estimate= -1401.63, SE= 451.70, $p=0.002$) were significantly associated with productive lifespan. Including all measures of lifetime RLTL dynamics in the same model and backwards eliminating all non- significant effects revealed that productive lifespan was most significantly influenced by the mean RLTL change. With mean RLTL change in the model, the mean absolute RLTL change became non-significant ($p>0.39$). More RLTL attrition was associated with a shorter productive lifespan (Figure 5).

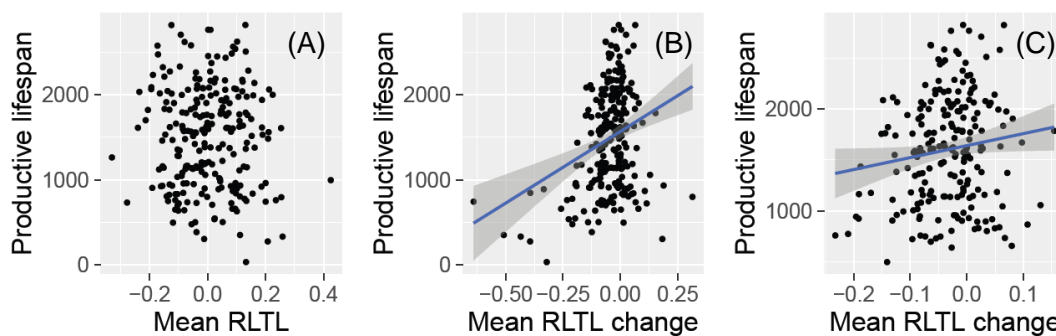


Figure 5: Relationship between measures of lifetime RLTL dynamics and productive lifespan. (A) No significant correlation between mean RLTL and productive lifespan ($p=0.44$). (B) Mean RLTL change was significantly associated with productive lifespan ($p<0.001$). (C) The relationship between mean RLTL change and productive lifespan remained statistically significant when animals with less than three samples were removed from the analysis ($p=0.047$).

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The analysis of productive lifespan in association with the three measures of lifetime RLTL dynamics was repeated for animals that had at least three RLTL measurements available to reduce the risk that results were confounded with the number of samples and young age at death. The association between mean RLTL change and productive lifespan remained statistically significant (Estimate: 1175.61, SE=588.49, $p=0.047$; Figure 5 C). Mean RLTL and mean absolute RLTL change had no statistically significant effect on productive lifetime ($p=0.63$ and $p=0.24$).

5.5. Discussion

In the present study animals varied in their amount and direction of mean RLTL change over life. RLTL shortening events were more frequent and bigger in magnitude than RLTL elongation events. This is in accordance with other longitudinal studies that have reported telomere lengthening in certain individuals (Bize et al., 2009; Chen et al., 2011; Farzaneh-Far et al., 2010; Gardner et al., 2005; Huzen et al., 2014; Kark et al., 2012; Nordfjäll et al., 2009; Shalev et al., 2012; Steenstrup et al., 2013; Svenson et al., 2011). Some authors claimed that telomere elongation observed in those studies was due to measurement error and short follow-up periods (Steenstrup et al., 2013), while others argued that it reflects a biological phenomenon (Bateson and Nettle 2016). We contend that it is time to seriously consider and investigate telomere elongation as an important biological process and discuss three plausible biological mechanisms that could cause a within-individual increase of telomeric DNA in a leukocyte sample. First, on the presence of stress or inflammation the composition of circulating leukocytes in the blood stream might change. Different leukocyte subpopulations have been shown to vary in both telomere length and telomere dynamics with age (Aubert, Baerlocher, et al. 2012; Baerlocher et al. 2007; Kimura, Gazitt, et al. 2010). For instance, granulocytes have longer TL on average than lymphocytes in adults (Aubert, Baerlocher, et al. 2012) so if an immune response elicited an increase in the granulocyte to lymphocyte ratio, average leukocyte TL would increase over time. Secondly, lymphocytes have been shown to express telomerase, particularly in the presence of a systemic inflammation (Weng et al. 1997; Valenzuela 2002; Hiyama et al. 1995; Akbar & Vukmanovic-Stejic 2007). The elongation of lymphocyte telomeres might increase average measurements of leukocyte TL. Thirdly, leukocytes ultimately descend from hematopoietic stem cells in the bone marrow, which are known to be dormant for a considerable time until activation (Baldrige et al., 2010; Essers et al., 2009; Trumpp et al., 2010; A. Wilson et al., 2008). The

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activation of a new pool of such stem cells with relatively long telomeres might cause an increase in the mean TL in circulating leukocytes. Measurement of changes in the leukocyte composition at the same time as measurement of TL, detection of telomerase expression in circulating leukocytes and measurement of TL in dormant and active hematopoietic stem cells might help to identify mechanisms of telomere elongation in leukocytes.

If telomere lengthening is assumed to reflect biological processes the next logical questions are: What factors trigger telomere elongation and what consequences does telomere lengthening have? In the present study we tested the effects of a variety of possible predictors on RLTL change and investigated if RLTL change affected productive lifespan. Most possible predictors proved to have no statistically significant effect on RLTL change (genetic group, feed group and birth year of the animal, and the occurrence of a health event within two weeks of sampling). Interestingly, even the time interval between consecutive samplings was not statistically significant ($p > 0.05$) indicating that the rate of RLTL change is highly variable. The only significant predictor of RLTL change was age: animals showed more telomere attrition within the first year of life than at older ages which is in accordance with results presented in the previous two chapters of this thesis. While early telomere attrition at a population level has been reported in the previous two chapters of this thesis, this result demonstrates that the early life decline in RLTL is driven by telomere shortening within individuals and not by selective disappearance of animals with long telomeres. In a study on Soay sheep telomere shortening within the first few months of life was also reported to be caused by intra-individual changes (Fairlie et al. 2015). Overall, telomere shortening in the beginning of life seems to be the strongest agreement between telomere dynamics in different vertebrate species (Baerlocher et al. 2007; Fairlie et al. 2015; Frenck et al., 1998).

Even though telomeres and their length dynamics have been intensely studied in humans, it is unclear if the rank order of individuals is fixed (Benetos et al. 2013) or heavily influenced by environmental factors (Blackburn et al., 2015). In the present study we offer evidence that: Consecutive RLTL measurements correlate moderately within individuals ($p < 0.001$) and individual RLTL measurements are affected by the previous RLTL measurements ($p < 0.001$). This suggests that animals with long RLTL shortly after birth are likely to maintain longer RLTL over their life. However, the correlation coefficient of consecutive RLTL measurements calculated

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in the present study is a lot lower ($r=0.38$) than the correlation coefficient of consecutive TL measurements calculated by Benetos et al. (2013) for humans ($r=0.9$). This, combined with the result that RLTL change is not repeatable within individuals, suggests that environmental factors impact individual RLTL dynamics in cattle considerably. In humans many lifestyle factors such as socio-economic status, psychological stress, smoking and body mass index are known to influence telomere length (Cherkas et al., 2008; Epel et al., 2009; Huzen et al., 2014; Kim et al., 2009; Shalev et al., 2012). Also, in animal studies chronic infection (Asghar et al. 2015) and exposure to stressful situations (Nettle et al. 2013; Reichert et al. 2014) have been linked to telomere shortening. It is largely unknown which factors might influence the change of telomere length in dairy cattle. However, one study reported bovine telomeres shortened over the course of lactation (Laubenthal et al. 2016), which indicates that the high metabolic demands during lactation may cause a stressful internal environment which manifests itself in telomere shortening.

Next to the predominant opinions about human telomere dynamics (fixed rank vs. largely influenced by environmental factors), there is the proposal of a more pulsatile change of TL in the literature (Svenson et al. 2011). After several studies in humans observed that TL change is more obvious when the sampling interval is small (Steenstrup et al., 2013; Svenson et al., 2011), Steenstrup et al. (2013) argued that this was proof for lengthening being mainly explained by measurement error. However, Svenson et al. (2011) proposed that TL might not change linearly but may oscillate across time. Therefore, we tested if the absolute amount of telomere change as a measure of different amplitudes of telomere oscillation was repeatable but found only weak support in our dataset (repeatability= 0.04). However, a sampling interval of approximately one year on average like in the present study might be too long to effectively investigate telomere oscillation. To measure the amplitude of oscillating change it must be guaranteed that the most extreme measurements are detected. A longitudinal study with shorter sampling intervals would be necessary to test this further.

In addition to investigating the factors affecting RLTL dynamics, the present study also addressed the possible biological implications of different RLTL dynamics. It was investigated if different measures of lifelong RLTL dynamics affected the productive lifespan of the animals. Productive lifespan describes the time from birth to culling and differs from “longevity” or “life span” in human or wild animal studies

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because the animals are culled for reasons relating mainly to health, fertility and productivity. However, productive lifespan is still biologically meaningful, because it reflects the ability of the animal to remain healthy, fertile and productive. Furthermore, productive lifespan largely dictates the profitability of the farm and the sustainability of the dairy sector. Mean RLTL was not significantly associated with productive lifespan ($p > 0.05$). However, the amount and direction of RLTL change affected productive lifespan significantly: More RLTL attrition was associated with poorer productive lifespan ($p < 0.001$). This association remained statistically significant ($p = 0.047$) after all animals with less than three samples were excluded from the analysis to rule out that the association between RLTL attrition and productive lifespan is only driven by physiologically fast RLTL depletion in early life. Our results agree with a study in jackdaws that showed that telomere loss affected post-fledging survival (Boonekamp et al. 2014). However, in the study on jackdaws only two TL measurements were taken in early life for each individual. Another study on Seychelles warbles focussed on adult samples only and found that both TL and telomere attrition were associated with survival (Barrett et al. 2013). In this study 36 out of 204 individuals were measured more than twice, but the majority had only one (110) or two (58) telomere length measurements. A third study on telomere dynamics in birds found that telomere attrition over five years in adult life predicted survival in Alpine swifts (Bize et al. 2009). However, the longitudinal dataset only included 22 individuals with two samples each. Also, a human study that was based on two telomere measurements taken five years apart from 608 individuals with coronary heart disease showed that telomere attrition was associated with a higher risk of mortality compared to telomere maintenance and telomere elongation (Goglin et al. 2016). Those studies strongly suggest that telomere attrition has biological consequences.

The present study is probably based on the most detailed longitudinal dataset investigating telomere change to date. It is also the first study that shows that an association of telomere attrition with lifespan also applies to the dairy industry sector where animals rarely die from natural causes. This is the first study that shows that more telomere attrition is associated with productive lifespan.

The amount of RLTL change is not repeatable implying that it is impossible to breed for animals that exhibit little telomere change and therefore have a superior productive lifespan. RLTL change in dairy cattle seems to be entirely caused by

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environmental factors which might make it attractive as a biomarker for the physiological stress an animal experiences. It might be possible to use RLTL change for the identification of factors that have unknown adverse effects on the animals' health. RLTL change may be usable as a biomarker for monitoring animal welfare (Bateson 2016) and as an optimisation tool for the management of dairy cattle. Further studies with well recorded environmental effects and phenotypes of diseases recorded throughout life are required to confirm if RLTL is an adequate biomarker for productive lifespan or animal welfare in dairy cattle (Brown et al. 2012; Bateson 2016). It also needs to be tested, if the inherited telomere length at the ages of one and five years as reported in Chapter 3 or the accumulated telomere attrition over life is the better predictor of productive lifespan. Also, the effect of milk yield on telomere change needs to be investigated.

Chapter 6

General discussion

6.1. Introduction

TL has been proposed as a predictive biomarker for productive lifespan (Brown et al. 2012) and animal welfare (Bateson 2016) in livestock. At the same time, very little is known about telomere dynamics and the genetics of TL in those species. Better understanding of TL, changes in TL over time and factors that influence both is necessary to characterise TL and explore its usability as a biomarker in animal breeding and animal husbandry.

The objectives of this thesis were 1) the development of robust methods for the measurement of bovine leukocyte TL in cattle, 2) the examination of change in bovine leukocyte TL across time at a population level and at an individual animal level, 3) the estimation of genetic parameters of bovine leukocyte TL and 4) the assessment of the association between bovine leukocyte TL profiles across time with productive lifespan of dairy cattle.

6.2. Thesis overview

In Chapter 2 a robust qPCR based method for the measurement of RLTL in cattle was developed. The effect of different DNA extraction methods on RLTL measurements was tested and it was found that silica based methods may alter RLTL measurements. However, it was possible to correct for the DNA extraction method effect by extracting the calibrator sample in the same way as the samples in the study. In Chapter 3 we called such a calibrator a “method-specific calibrator”. As a consequence, quick silica based DNA extraction methods were deemed to be suitable for qPCR based telomere studies which has the potential to improve the sample throughput considerably.

In Chapter 3 RLTL dynamics with age were investigated at the population level using a cow dataset with samples from birth to culling (1328 samples of 308 animals) and a calf dataset with more frequent samples within the first year of life (284 samples of 38 calves). RLTL declined within the first year considerably but

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remained relatively stable thereafter. Different factors were investigated for their effect on RLTL using mixed models. Age significantly affected RLTL ($p < 0.001$), but also birth year ($p = 0.004$, cow dataset) and birth season ($p = 0.04$, calf dataset) and birth weight ($p = 0.04$, calf dataset) influenced RLTL. Using pedigree information, overall heritability estimates of 0.32 (SE=0.08) and 0.38 (SE=0.03) were calculated for the calf and the cow dataset respectively. This implies that RLTL is a trait that is significantly shaped by the genetics of the animal and inherited from the parental generation. In addition, environmental factors such as those associated with the birth year and birth season of the animal influence RLTL. Finally, RLTL at different ages was analysed in relation to productive lifespan using Cox proportional hazard models and the results showed that RLTL at the ages of one and five years was associated with better survival in the dairy herd. Animals with long telomeres at those ages survived for longer ($p < 0.05$).

In Chapter 4 the individual shape of RLTL change within individuals of the cow dataset was analysed using random regression models. These models account for the covariance between repeated records of the same animal. A fixed curve which illustrates RLTL dynamics for the whole population showed similar results as reported in Chapter 3: RLTL shortened in the beginning of life and remained relatively stable afterwards. Individual RLTL change profiles were calculated as the deviation from the fixed curve and proved to be significantly different between individuals ($p < 0.05$). Differences in profiles were mainly due to a difference in the intercept (mean RLTL over life) and less due to different shapes of curves (RLTL change pattern). Even though individual curves differed, no significant relationship between the shape of the curve and productive lifespan was found. Random regression models were also used for the calculation of heritability estimates over the sampling period. Heritability was relatively high (0.36 - 0.47) (SE= 0.05-0.08) and close to constant over life. The genetic correlation of RLTL between subsequent time points decreased from close to unity to 0.69 (maximal SE = 0.087) indicating that bovine RLTL in early life might be partially under different genetic control than RLTL later in life.

In Chapter 5 within individual RLTL change was further explored. Because individual animals curves calculated with random regression models in Chapter 4 might be too restrictive to capture more pulsatile RLTL change, the latter was calculated as the difference between two successive RLTL measurements that were both pre-

adjusted for qPCR plate and qPCR row to reduce a part of the known measurement error. Using a mixed model it was shown that age affected the amount of RLTL change significantly ($p < 0.001$): Young animals shortened their telomeres considerably more than older individuals. In addition to RLTL shortening, lengthening of RLTL was observed between two consecutive measurements in some individuals. Overall, successive RLTL measurements correlated moderately with each other ($r = 0.38$, $p < 0.001$) and each RLTL measurement was significantly influenced by the previous measurement ($p < 0.001$). To investigate the biological implications of TL and the amount and direction of change in TL, three lifetime measures of RLTL dynamics (mean RLTL, mean RLTL change and mean absolute RLTL change) were developed and their association with productive lifespan was investigated. It was found that mean RLTL over life did not affect productive lifespan significantly ($p > 0.05$), but mean RLTL change was significantly associated with age at culling ($p < 0.001$).

The present chapter will discuss the results of the previous four chapters with the aim to demonstrate how this thesis contributes to the knowledge about telomeres and their dynamics in vertebrates in general and in livestock species in particular. The discussion will also point out open questions that require more investigation. Each thesis objective will be discussed separately below.

6.3. The development of robust methods for the measurement of bovine leukocyte TL in cattle

For this thesis a large scale telomere study was planned which required relatively fast and cost effective methods for TL measurement. Furthermore, the work presented was considered to be the first step towards exploring RLTL as a biomarker with possible application in the dairy industry. Such measurements needed to be quick and affordable, because even the best predictor of productive lifespan would not be useful as a biomarker for the livestock industry if it was not cost-effective. Therefore, an existing qPCR assay for the measurement of RTL in humans (Cawthon 2002) was adapted for the measurement of RLTL in cattle. Throughout this thesis the constant noise emanating from the qPCR method was reduced by measuring all samples in triplicate and by ensuring high integrity and purity of all DNA samples that were included on qPCR plates. To enable a more objective judgement of DNA integrity which is typically estimated by running gel electrophoresis of genomic DNA and by observing “a clear band” on the gel, a

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numeric gel score was developed. The numeric gel score makes the evaluation of integrity gels more objective, easier to compare between samples and studies, and simpler to include in statistical models for data analysis.

While the qPCR assay for the measurement of bovine telomere length quickly delivered robust results (correlation between repeated plates >0.88, repeatability: 0.88), the choice of a suitable DNA extraction method required further consideration. In qPCR based telomere studies, DNA extraction and quality control are often the time limiting steps. Therefore, a higher sample throughput at the extraction stage would accelerate the whole project and allow more detail in longitudinal studies. The need for faster sample processing in general and faster DNA extraction techniques in particular was obvious from the steep increase of published studies that used qPCR for telomere length measurement and silica membrane based methods for the extraction of DNA (Chapter 1, Figure 1 and Appendix A, Table 1). Silica based DNA extraction methods are known to be fast. They are typically available in two designs: spin columns and 96 well spin plates. A physical barrier made of silica is used for specifically binding DNA, washing all substances that are not required away and collecting DNA in the end using an elution buffer. During elution, the DNA has to pass through the silica membrane. Many authors hypothesised DNA could be sheared either during centrifugation or during elution from silica membranes and the literature suggested that shearing of DNA might alter telomere length measurements (Denham et al. 2014; Cunningham et al. 2013; Hofmann et al. 2014; Tolios et al. 2015). However, it was not clear, why telomere length measurement by qPCR should be affected because qPCR measures not telomere length but the total amount of telomeric DNA in a sample. It makes no difference if telomeres are present in one piece or in several smaller pieces as long as not too many primer binding sites are compromised. However, in Chapter 2 a DNA extraction method effect on RLTL was detected which was in accordance to other published studies (Denham et al. 2014; Cunningham et al. 2013; Hofmann et al. 2014; Tolios et al. 2015). When RLTL was calculated using a calibrator sample that was extracted with a non-silica method, silica extracts appeared to have less telomeric DNA. A possible explanation could be that telomeres are indeed sheared while bound to silica membranes and shorter sequences of telomeric DNA are removed from the sample during washing steps. Further studies are required to better understand how silica membranes alter telomere length measurements. It might be possible to test the

washing buffers after they ran through the silica membranes for the presence of short DNA which would support the hypothesis above.

However, it was also shown in chapter 2 that it was possible to correct for the DNA extraction method effect on RLTL simply by extracting the calibrator sample using the same method as for the samples in the study (“extraction method specific calibrator”). By doing this, RLTL measurements from silica extracts were not only directly comparable to those from Puregene extracts, the results of Chapter 2 also showed that silica based methods delivered more repeatable RLTL measurements than the salting out extraction method. This might be due to a more homogeneous solution of the DNA. Combined with the facts that silica based methods were faster, cheaper and needed less post extraction purification of samples, they seemed to be the most appropriate DNA extraction method for the large scale telomere project presented in this thesis.

It has to be tested individually, if silica membranes are appropriate for the use in other telomere projects (different labs, different species). Also, silica membranes may not be suitable for telomere length measurement using other methods such as TRF. While qPCR measures the absolute amount of telomeric DNA within a sample, TRF measures actual telomere length. The integrity gels shown in Figure 1 of Chapter 2 suggest that DNA integrity is on average better for DNA samples that were extracted using the salting out method. Also, RLTL measurements that were calculated using the same calibrator for all extraction methods suggest that silica membranes in some way alter telomeric DNA. Even though it was possible to correct for this effect on qPCR by using a DNA extraction method specific calibrator, the same might not be true for TRF.

In the end, the right method for DNA extraction and telomere length measurement has to be chosen individually based on the purpose and requirements of the study. For this thesis a considerable amount of time and work was spent on choosing the most appropriate lab methods for the purpose of measuring bovine telomere length in more than 1,500 samples. The combination of a silica based DNA extraction method and qPCR for telomere length measurement produced the best results for the present study.

6.4. The examination of change in bovine leukocyte TL across time at a population level and at an individual animal level

In this thesis several approaches were used for the investigation of RLTL dynamics with age: 1) In Chapter 3 RLTL was described at the population level and it was observed that RLTL shortens on average within the first year of life and remains relatively stable thereafter. 2) In Chapter 4 random regression models were used to describe RLTL change across time at the population level and RLTL change within the individual animal across time as deviation from the average population curve. Chapter 4 showed that animals differed significantly in their telomere change profiles and most of the difference was due to a difference in the intercept (average RLTL over life) while less variance between animals was due to different shapes of individual RLTL change curves. 3) In chapter 5 three lifetime RLTL measurements were calculated: Mean RLTL, mean RLTL change and mean absolute RLTL change. This chapter proved that RLTL attrition early in life (as observed in Chapters 3 and 4) was due to within individual shortening of telomeres and not due to selective disappearance of animals with long telomeres. It also showed that animals differ in the amount and direction of telomere length change.

The lack of telomere attrition in adult life in this thesis that has been observed before in cattle (Miyashita et al. 2002; Brown et al. 2012) could be explained by the relatively young age of even the oldest animals in the present study. Even though animals included in this thesis lived a typical life of a dairy cow in production, compared to a theoretically possible lifespan of approximately 20 years for cattle, six year old animals can probably be compared to young adults in humans. It is possible that there is a plateau between the ages of one and six years that is followed by a second decline of telomere length at older ages comparable to the dynamics that were observed in humans (Frenck et al. 1998). There are animals that are as old as thirteen years at the Crichton Royal Farm that possess longitudinal samples from a juvenile age onwards. They were excluded from studies presented in this thesis because they did not have an early life blood sample present that was important for the purpose of this study. It would be interesting to measure TL of those animals to better understand telomere dynamics in older cattle.

Another possible explanation for the maintenance of TL in adult life is the possible expression of telomerase in leukocytes. Particularly lymphocytes are known for their

ability to express telomerase (Weng et al. 1997; Valenzuela 2002; Hiyama et al. 1995; Akbar & Vukmanovic-Stejić 2007) and lymphocytes make up the majority of leukocytes in bovine blood. It would be interesting to conduct a more detailed study in adult life, where not only telomere length but also leukocyte composition and telomerase expression is measured at the same time. There are two studies that show that across vertebrate species telomerase activation is negatively associated with body mass (Gomes et al. 2011; Seluanov et al. 2007). From those studies it would be expected that cattle repress telomerase and Gomes et al. (2011) indeed did not detect telomerase expression in adult cattle. Telomerase expression in bovine blood samples merits future more detailed studies.

Individual animal RLTL dynamics vary considerably which was shown in Chapters 4 and 5. The results of Chapter 4, where random regression models were used for the investigation of different RLTL dynamics, showed that most of the difference between individual animal curves of RLTL change over age is due to a difference in the intercept (average RLTL over life). A smaller proportion of the variance between animals is explained by different shapes of curves. The difference in average RLTL over life might be influenced by the presence of interstitial telomeric sequences which are telomeric repeats (TTAGGG) that are not located at the ends of chromosomes but between telomeres and centromere (Foote et al., 2013; Nanda et al., 2002; Ruiz-Herrera et al., 2002). The amount of interstitial telomeric DNA typically varies considerably between individuals of the same species but is constant across tissues within the same individual (Lin and Yan 2008). Interstitial telomeres are constant in their length over life. It is not clear if cattle possess interstitial telomeres and if they vary in their amounts of such sequences. The only study that investigated the presence of interstitial telomeres in the cattle genome used fluorescence in situ hybridisation (FISH) for telomere sequence detection and found no evidence for telomeric sequences at interstitial sites (De La Señá et al., 1995). However, this only shows that cattle are not likely to possess long stretches of interstitial telomeres in their genome. But FISH is not sensitive enough to detect short interstitial telomeric sequences (Mohan et al. 2011; Bolzán & Bianchi 2006) and it is therefore unknown if those short telomeric sequences are present in the cattle genome. TRF images published by Miyashita et al. (2002) that show bands at low molecular weight for a few animals in their study may suggest the presence of interstitial telomeres in the cattle genome.

With qPCR the total amount of telomeric DNA is measured, regardless of its location which results in a measurement of the sum of telomeric DNA at the end of chromosomes and at interstitial regions. There are methods described for the measurement of interstitial telomeres: 1) a TRF based method that compared average telomere length of the same sample once measured with the in gel and once with the traditional TRF method (Foote et al. 2011). This approach showed that 15-45% of telomeric sequences in birds consisted of interstitial telomeres (Foote et al. 2011). 2) A possible method for the measurement of interstitial telomeres by qPCR is to measure the total amount of telomeric DNA (like in this thesis), to digest telomeric DNA at the ends of chromosomes using the exonuclease BAL-31 (Burr et al. 1992; Mohan et al. 2011) and to repeat the measurement of telomeric DNA with qPCR. The second qPCR measurement should only detect interstitial telomeres. The first measurement minus the second measurement informs about telomere length at the ends of chromosomes. Furthermore, whole genome sequencing is performed for the majority of the cattle kept at the Crichton Royal farm. Even though the detection of repetitive DNA such as telomeric sequences is not accurate, searching for telomeric sequences at interstitial sites may inform about the variance in the amount of interstitial telomeres between animals.

To better understand telomere length dynamics in cattle, it would be interesting to measure TL without the inclusion of interstitial telomeres in future experiments. Also, it would be interesting to investigate further factors that may influence TL dynamics at young and old ages in more detail. Candidates for such factors might be health events, stress within the herd (number of animals, hierarchy), food quality, temperature and weather.

6.5. The estimation of genetic parameters of bovine leukocyte telomere length

In this thesis the first heritability estimates of RLTL in a livestock species are presented. In Chapter 3 an overall heritability of 0.32 (SE=0.08) and 0.38 (SE=0.03) was calculated for the calf and cow dataset respectively. Random regression models were used in Chapter 4 to explore the change of heritability across age. Using random regression models heritability estimates for the cow dataset were slightly higher but statistically not different from those calculated in Chapter 3 (0.36 - 0.47) (SE= 0.05-0.08). Heritability estimates were relatively stable over life indicating that it is possible to influence RLTL at all ages with selective breeding. However, the

usefulness of breeding animals with a particular RLTL phenotype has yet to be proven. Also, the possible presence of interstitial telomeres in the cattle genome may have led to an over-estimation of the heritability of the amount of telomeric DNA at the end of chromosomes. However, only the amount of telomeric sequence at the ends of chromosomes is believed to influence health and longevity in humans and animals. It would be interesting to repeat the calculation of heritability estimates for RLTL that was corrected for the presence of interstitial telomeres.

While RLTL was shown to be heritable in Chapters 3 and 4, the amount and direction of RLTL change with time is not heritable and therefore mainly affected by the environment. Further studies are needed to determine the environmental factors that cause a change in RLTL in dairy cattle.

In Chapter 4 random regression models were also used to calculate the genetic correlation of RLTL across time which decreased from near unity to 0.69 (maximal SE = 0.087). This implies that RLTL in early life may be partially under a different genetic control than RLTL later in life. More studies are required to identify causal genes affecting RLTL and RLTL change. Genome wide association studies and post-genomic functional analyses would be helpful in this regard.

6.6. The assessment of the association between bovine leukocyte telomere length profiles across time with productive lifespan of dairy cattle

In this thesis, several TL related measurements were investigated regarding their relationship with productive lifespan. Productive lifespan was defined as the time from birth to culling in days and differs from life span or longevity in humans and wild animals because of culling being a non-natural cause of death. However, it is still biologically meaningful because animals are usually culled for poor fertility and diseases. Improving productive lifespan equals improving the health span of dairy cows while keeping them productive.

In Chapter 3, RLTL at the ages of zero to six years were analysed in relation with productive lifespan using cox proportional hazard models. RLTL at the ages of one and five years were positively and significantly associated with productive lifespan ($p < 0.05$). At the moment, it is unclear why RLTL is associated with productive lifespan at these specific ages only. However, that the relationship is not constant

Chapter 6: General discussion

across all ages shows that the rank order of the animals regarding their RLTL is not maintained over life.

In Chapter 4 the shape of the random curve was analysed in relation with productive lifespan but no significant relationship was found ($p > 0.05$). Because the difference in the shape of curves was predominantly due to the differences in the intercept it was not surprising that the mean RLTL over the whole sample period was also not associated with productive lifespan in Chapter 5. However, in separate models mean RLTL change and mean absolute RLTL change were both significantly associated with productive lifespan ($p < 0.001$ and $p = 0.002$, respectively). Testing all measures of lifetime RLTL dynamics in the same model revealed that mean RLTL change was the best predictor of productive lifespan. Animals with more than average telomere attrition were culled at a younger age.

As mentioned above RLTL change was not heritable and is therefore mainly influenced by environmental factors. In this thesis only age could be identified as a factor that influenced the amount and direction of change. Further studies are required to identify other environmental influences to explore if RLTL change is caused by stimuli that might be interesting for the detection of animal welfare problems. For future studies it would be interesting to establish a close monitoring of changes in telomere length while recording data on both environmental measures (temperature, precipitation, group size, etc.) and health related measurements (body temperature, heart rate, leukocyte count, etc.). It might be possible to use milk samples as an easily accessible tissue for telomere length measurement in cows. Telomere length in milk might be closely correlated to leukocyte telomere length, because somatic cells in milk samples mainly consist of filtrated leukocytes. If it was possible to detect health issues of the animal by the amount of telomere change, before clinical signs are recognisable, it might be possible to use telomere change measurements as a biomarker for animal welfare issues and as a tool for the optimisation of the farm management. Future studies are required to test the potential of RLTL change for these purposes further

6.7. Strengths and novelty of this study

The most obvious strength of the present study is the rather unique longitudinal dataset that allows the investigation of change in TL from shortly after birth through adolescence and adult life to culling of the animals. So far, this is the biggest

longitudinal study on telomere length dynamics outside the human literature and the first study with enough statistical power for random regression analysis of TL. The sampling period spans the normal productive lifespan of dairy cattle which means this study can be described as a lifelong study for this species. Most longitudinal studies in humans and other vertebrates usually include only two samples per individual and span only a short period of the total possible lifespan of the study organisms. Only a few studies so far such as a study on captive zebra finches and wild Soay sheep were lifelong longitudinal studies (Heidinger et al. 2012; Fairlie et al. 2015), but included fewer animals and samples than the studies presented in this thesis. Animals for this thesis were kept at a research farm which allowed careful monitoring of phenotypes. Outside the human literature there are not many genetics studies on TL and this thesis contributes to a better understanding of the genetics of RLTL and its role in dairy cattle longevity.

6.8. Conclusion

This thesis contributed to a better understanding of TL dynamics with age in dairy cattle. The first heritability estimates of RLTL in a livestock species were presented and first evidence is shown that RLTL at specific ages and RLTL change over the sample period is associated with the productive lifespan of dairy cows. Further studies are required to clarify to what degree those results were influenced by the presence of interstitial telomeres. Also, future studies are required to investigate the optimum means of incorporating outcomes of the present study in breeding programmes and on-farm monitoring practices aiming to enhance animal longevity.

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Appendix A

Table 1: Telomere length measurement and DNA extraction methods in studies of non-human and non-model organisms

Author and year of publication	Species	DNA extraction method	Extraction Principle	Telomere length measurement method
Miyashita et al. 2002	Cattle	SDS-phenol-proteinase K extraction	organic extraction method	TRF
Hausmann et al. 2003	Birds	salt extraction, alcohol precipitation method & CHEF agarose plugs (Biorad, Hercules, CA, 2003 samples)	salting out & agarose plugs	TRF
Betts et al. 2005	Goats	UNKNOWN	UNKNOWN	TRF
Hausmann et al. 2005	Tree swallows	salt extraction, alcohol precipitation method & CHEF agarose plugs (Biorad, Hercules, CA, 2003 samples)	salting out & agarose plugs	TRF
Francis et al. 2006	Sea urchins	phenol/ chloroform	organic extraction method	TRF
Juola et al. 2006	Great Frigatebirds	alcohol-based DNA precipitation technique (H. L. Gibbs unpublished protocol)	UNKNOWN	TRF
Pauliny et al. 2006	Sand martins and dunlins	phenol/ chloroform	organic extraction method	TRF (in gel)
Scott et al. 2006	Alligator	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Alexander et al. 2007	Sheep	DNeasy Blood & Tissue kit (QIAGEN)	silica based	TRF
Baerlocher et al. 2007	Baboon	NONE	NONE	FISH
Jemielity et al. 2007	Ant	Genomic-tip columns (QIAGEN)	anion-exchange method	TRF
Bronikowski 2008	California garter snake	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Hatase et al. 2008	Loggerhead sea turtle	QuickGene Mini80 (Autogen)	nucleic acid extraction machine	qPCR
Hartmann et al. 2009	turquoise killifish	QIAmp (QIAGEN)	silica based	TRF & Q-FISH
Ujvari & Madsen 2009	Tropical Pythons	phenol/ chloroform	organic extraction method	TRF
Olsson et al. 2010	sand lizards	phenol/ chloroform	organic extraction method	TRF
Tilesi et al. 2010	Cattle	Wizard Genomic Purification kits (Promega)	salting out	TRF
Anchelin et al. 2011	Zebrafish	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF and Q-FISH
Beaulieu et al. 2011	Ade`lie penguins	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Benetos et al. 2011	Dogs	phenol/ chloroform	organic extraction method	TRF
Foote et al. 2011	Southern giant petrels	phenol/ chloroform	organic extraction method	TRF
Foote et al. 2011	Lesser black-backed gull	phenol/ chloroform	organic extraction method	TRF
Horn et al. 2011	Kakapo	QIAmp (QIAGEN)	silica based	TRF
Izzo et al. 2011	Australian sea lion	Puregene (Gentra Systems)	salting out	qPCR

Appendix A

Olsson et al. 2011	Lizard	phenol/ chloroform	organic extraction method	TRF
Olsson et al. 2011	Sand lizards	phenol/ chloroform	organic extraction method	TRF
Brown et al. 2012	Cattle	DNeasy 96 Blood & Tissue Kit (QIAGEN)	silica based	qPCR (multiplex)
Geiger et al. 2012	King penguin	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Godwin et al. 2012	Sydney rock oyster	phenol/ chloroform	organic extraction method	TRF
Hausmann et al. 2012	Bovans White chicken	0.8% agarose plugs	agarose plugs	TRF
Heidinger et al. 2012	Zebra finch	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Pauliny et al. 2012	Barnacle goose	phenol/ chloroform	organic extraction method	TRF
Plot et al. 2012	Leatherback turtles	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Turbill et al. 2012	Djungarian hamsters	Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich)	silica based	qPCR
Ujvari et al. 2012	Tasmanian devil	phenol/ chloroform	organic extraction method	TRF
Voillemot et al. 2012	Collared flycatcher	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Angelier et al. 2013	American redstart	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Barrett et al. 2013	A. sechellensis	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Bauch et al. 2013	Common tern	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Caprioli et al. 2013	Barn swallows	PureLink Genomic DNA extraction Kit (Life technologies)	silica based	TRF
Henriques et al. 2013	Zebrafish	phenol/chloroform and chloroform-isoamyl alcohol extraction	organic extraction method	TRF
Mizutani et al. 2013	Blacktailed gulls	DNeasy Blood & Tissue kit (QIAGEN)	silica based	TRF
Nettle et al. 2013	European Starling	NucleoSpin Blood Kit (MACHEREY-NAGEL)	silica based	qPCR
Reichert et al. 2013	Zebra finches	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Young et al. 2013	Thick-billed murre	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Aydinonat et al. 2014	Grey Parrots	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Bauch et al. 2014	common tern	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Beirne et al. 2014	European badgers	Fermentas spin columns (GeneJet)	silica based	qPCR
Boonekamp et al. 2014	Jackdaw	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Gruber et al. 2014	Arctica islandica	DNeasy Blood & Tissue kit (QIAGEN)	silica based	TRF
Guo et al. 2014	Epinephelus bruneus and Epinephelus moara	NONE	NONE	FISH
Herborn et al. 2014	European shag	NucleoSpin Blood Kit (MACHEREY-NAGEL)	silica based	qPCR
Reichert et al. 2014	King penguin	NucleoSpin Blood QuickPure Kit (MACHEREY-NAGEL)	silica based	qPCR

Appendix A

Reichert et al. 2014	Zebra finches	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Reichert et al. 2014	Zebra finches	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Schultner et al. 2014	Black-legged kittiwakes	CHEF Genomic DNA Plug kit (Bio-Rad)	agarose plugs	TRF
Tackney et al. 2014	Chimpanzees and Humans	QIAamp DNA Blood Mini Kit (QIAGEN)	silica based	qPCR (multiplex)
Asghar et al. 2015	great reed warblers	phenol/ chloroform	organic extraction method	qPCR
Badás et al. 2015	Blue tits	ammonium acetate protocol	salting out	qPCR
Hau et al. 2015	Eurasian blackbirds	0.8% agarose plugs	agarose plugs	TRF
Noguera et al. 2015	Zebra finches	DNeasy Blood & Tissue kit (QIAGEN)	silica based	qPCR
Rattiste et al. 2015	Common gulls	phenol/ chloroform	organic extraction method	qPCR
Sánchez-Guillén et al. 2015	Wild mouse	NONE	NONE	FISH
Bateson et al. 2015	starling	NucleoSpin Blood Kit (MACHEREY-NAGEL)	silica based	qPCR

Appendix B

Characterisation of the Crichton herd

Holstein Friesian dairy cattle for this PhD thesis were originally kept at the Langhill Farm close to Edinburgh, before the majority of the animals was moved to the Crichton Royal Farm in Dumfries in 2002. Because of its history the herd is still often referred to as the “Langhill herd”. At the Crichton Royal Farm 200 milking cows plus their calves and replacement heifers are kept at any time. One half of the milking cows belongs to a genetic line that has been selected for high milk protein and fat yield (S), while the other half is deliberately maintained on a UK average productivity level (C). Selection for those two genetic lines started in 1970. Although animals of the different genetic lines differ in productivity, one may not forget that dairy cattle in general are relatively highly inbred when compared to natural populations. Animals of the C and S line do not significantly differ in their frame, weight or body condition score ($p>0.05$) as determined using t-tests for the animals in the present study. Each new-born calf is weighed and earmarked and kept in an individual housing for the first few days of its life. Then C and S calves are transferred to igloos, where groups of calves live together. Their individual food intake is monitored. Until their first calving each heifer is kept in the same way. At first calving each cow is randomly allocated to a high forage (HF) or low forage (LF) diet. The LF diet is based on human food by-products and consists of a concentrate blend (biscuit meal, sugar beet pulp, breakfast cereal, wheat distillers dark grains, Hipro soya bean meal, chopped straw, whole crop wheat, protected fat (Megalac), liquid whey permeate and minerals mixed with water). Cows of the S and C line that are managed in the LF diet are housed together and continuously over the year without

Appendix B

a grazing period. The HF diet on the other hand is based on feed that is grown at the Crichton Royal Farm and consists of grazed grass, grass silage, red clover silage, forage maize, lucerne silage, crimped wheat, and beans, balanced with a purchased mineral. Two hectares of land have been allocated to each cow for feed production and grazing. Cows of the S and C line that are managed on the HF diet are kept together. They are turned out over the summer months for grazing and they are housed over the winter months. The food and water consumption of all cows is monitored.

All cows are milked three times daily. Milk yield and composition and the somatic cell count as an indicator for (subclinical) mastitis is recorded. Cows leave the milking parlour over a pressure plate for lameness detection.

General data on animal performance, behaviour, fertility and health are measured for each animal, while specific projects can require additional measurements such as methane gas emission. Also, most of the animals on the farm are either genotyped or whole genome sequenced.

Additionally to a broad spectrum of data, blood samples are taken regularly for the examination of immune traits and telomere length over life. Each animal is blood sampled shortly after birth and then annually in spring. Because of this sampling routine and because of calves being born all year round, age at sampling varies considerably for adult animals. Also, the sampling interval varies greatly. Figure 1 shows histograms that illustrate (A) the age at sampling of the animals, (B) the number of samples per animal, (C) the birth weight of the animals and (D) the weight distribution of adult animals.

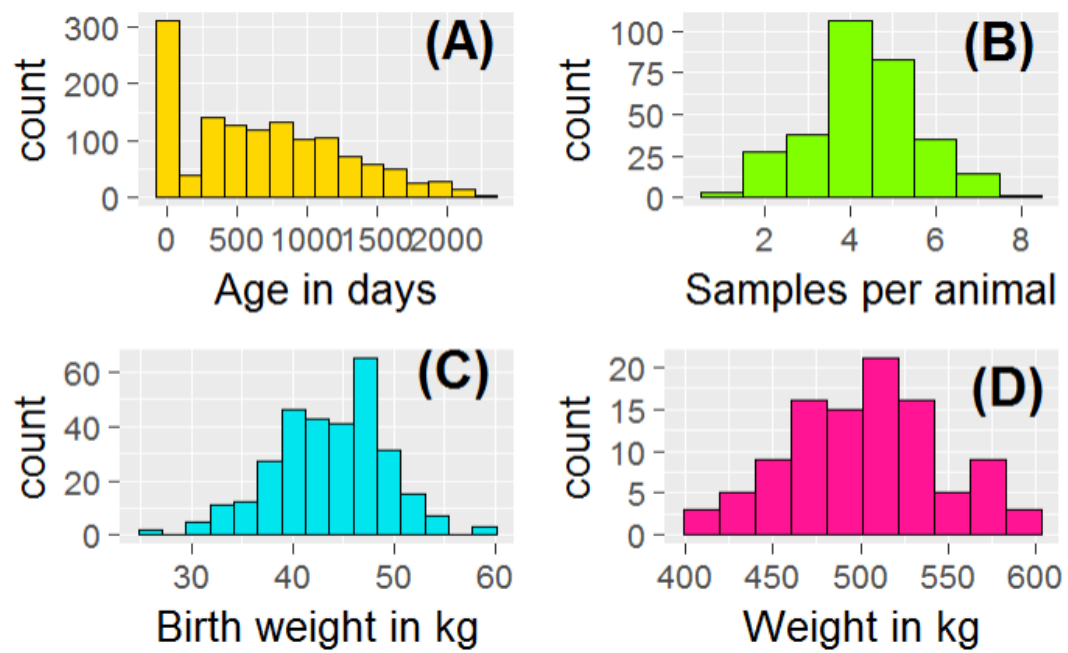


Figure 1: Histograms. (A) age at blood sampling in days, (B) number of blood samples/ telomere length measurements per animal, (C) birth weight and (D) adult weight distribution

Appendix C

Published Papers

This appendix contains published material arising from this thesis:

Seeker, L. A., Holland, R., Underwood, S., Fairlie, J., Psifidi, A., Ilska, J. J, Bagnall, A., Whitelaw, B., Coffey, M., Banos, G., Nussey, D. H. (2016). Method specific calibration corrects for DNA extraction method effects on relative telomere length measurements by quantitative PCR. PLoS ONE, 11(10), 1–15.
<http://doi.org/10.1371/journal.pone.0164046>

Seeker, L. A., Ilska, J. J., Psifidi, A., Wilbourn, R. V., Underwood, S. L., Fairlie, J., Holland, R., Froy, H., Bagnall, A., Whitelaw, B., Coffey, M., Nussey, D. H. and Banos G., “Longitudinal changes in telomere length and associated genetic parameters in dairy cattle analysed using random regression models,” PLoS One, pp. 1–15, 2018.

RESEARCH ARTICLE

Method Specific Calibration Corrects for DNA Extraction Method Effects on Relative Telomere Length Measurements by Quantitative PCR

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Data Availability Statement: Data is available at <https://laseeker.github.io/DNAextractionTelomeres/>.

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Abstract

Telomere length (TL) is increasingly being used as a biomarker in epidemiological, biomedical and ecological studies. A wide range of DNA extraction techniques have been used in telomere experiments and recent quantitative PCR (qPCR) based studies suggest that the choice of DNA extraction method may influence average relative TL (RTL) measurements. Such extraction method effects may limit the use of historically collected DNA samples extracted with different methods. However, if extraction method effects are systematic an extraction method specific (MS) calibrator might be able to correct for them, because systematic effects would influence the calibrator sample in the same way as all other samples. In the present study we tested whether leukocyte RTL in blood samples from Holstein Friesian cattle and Soay sheep measured by qPCR was influenced by DNA extraction method and whether MS calibration could account for any observed differences. We compared two silica membrane-based DNA extraction kits and a salting out method. All extraction methods were optimized to yield enough high quality DNA for TL measurement. In both species we found that silica membrane-based DNA extraction methods produced shorter RTL measurements than the non-membrane-based method when calibrated against an identical calibrator. However, these differences were not statistically detectable when a MS calibrator was used to calculate RTL. This approach produced RTL measurements that were highly correlated across extraction methods ($r > 0.76$) and had coefficients of variation lower than 10% across plates of identical samples extracted by different methods. Our results are consistent with previous findings that popular membrane-based DNA extraction methods may lead to shorter RTL measurements than non-membrane-based methods. However, we also demonstrate that these differences can be accounted for by using an extraction method-specific calibrator, offering researchers a simple means of accounting for

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differences in RTL measurements from samples extracted by different DNA extraction methods within a study.

Introduction

Telomere shortening has recently been identified as one of nine ‘hallmarks of aging’ [1] and blood cell telomere length (TL) is an increasingly widely measured biomarker in human epidemiology and vertebrate ecology [2–4]. Many methods are available to measure TL, each with their own strengths and drawbacks [5,6]. Quantitative PCR (qPCR)-based methods have become increasingly popular in recent years, presumably due to their being faster, cheaper and requiring less DNA than most other methods [5,6]. However, the qPCR method has drawbacks, notably a lower repeatability compared to terminal restriction fragment (TRF) southern blot [7,8] and the relative units of measurement, which makes comparison across studies and species extremely challenging [5,7] if not impossible. Furthermore, there is mounting recent evidence that relative TL (RTL) measurements by qPCR may be influenced by methods of sample acquisition and storage [9] and DNA extraction methods [10–14]. Understanding how such methodological variation may influence RTL measurements by qPCR both within and among laboratories is essential for evaluating and comparing results of telomere studies.

A central requirement of all methods of TL measurement is the extraction of a suitable quantity of high quality DNA. A considerable number of DNA extraction methods have been employed to date by researchers studying TL [10]. In general two different types of DNA extraction methods can be distinguished: One uses a solid phase such as silica membranes or magnetic beads. DNA binds to the solid phase, is washed and then eluted. The other type is based on the transition of DNA between different solvents. Those methods (for example salting out or phenol-chloroform extractions) do not require a solid phase. The question that arises from the literature is whether solid phases act as physical barriers that shear DNA and therefore cause shorter TL measurements. Two recent studies using human blood samples with the qPCR method suggested that silica membrane-based DNA extraction methods yield shorter RTL measurements than other methods [10,11]. Two further studies have reported differences in mean TL from DNA extracted using a range of different methods, although these differences were not specifically linked to the use of silica membranes [12,13]. Recently, another study found that RTL from samples extracted by a magnetic bead method was shorter when compared to salting out and phenol chloroform [14]. Although it is obviously desirable to keep methodology as consistent as possible, potentially valuable and informative archived DNA samples may be available to researchers interested in telomere dynamics which may not have been extracted by the same technique. In such cases, understanding and potentially accounting for the effects of extraction method on TL measurement is essential [15]. Furthermore, a better understanding of such methodological effects could help ensure appropriate aspects of DNA preparation methodology are accounted for in meta-analyses of TL studies [10].

The qPCR method measures RTL as the total amount of telomeric sequence relative to the amount of a non-variable copy reference gene sequence within the same DNA sample [16]. Standard methods for calculating RTL require a calibrator sample (also called “reference sample” [16] or “golden sample” [6]), which is an identical DNA sample included on every qPCR plate for both telomere and reference gene reactions. Sample RTL is expressed relative to the calibrator to account for random measurement error and resulting plate-to-plate variation. A wide range of samples have been used as calibrators: DNA from a chosen individual, pooled

DNA from several individuals [16] or commercially available DNA [14]. Previous studies examining effects of DNA extraction method on RTL appear to have used a single calibrator, extracted by one identical method [10–14]. They observed extraction method dependent differences in RTLs that in some studies appear to be not random but systematic [10,11,14]. In principle, it should be possible to account for such systematic extraction method effects by taking the same calibrator sample and extracting DNA from it using different methods to match the methods used on the samples in the study. With this approach, the calibrator should be influenced in the same direction and to a similar degree by the extraction method. Using such a DNA extraction method specific calibrator in RTL calculations, could therefore adjust for any effect of extraction method on the samples' telomere length. The effectiveness of this approach has yet to be tested.

The objective of the present study was to assess the effect of two different DNA extraction methods, and the use of different calibrators on RTL measurements. We compared RTL measurements of blood samples that were collected from a Holstein Friesian cattle population after extracting DNA using two silica membrane-based DNA extraction protocols and a salting out (non-membrane-based) method. To validate our results with samples from a different species we compared one of the two silica membrane-based methods with the salting out method using buffy coat samples from wild Soay sheep. We found high repeatability of RTL measurements, regardless of DNA extraction method, and no difference in mean RTL among extraction methods when a DNA extraction method specific (MS) calibrator was used.

Materials and Methods

Study systems & sampling

Whole blood samples were collected from Holstein Friesian cattle during 2009–2013 at the Crichton Royal Farm (Dumfries, Scotland) as part of a long-term genetics study for which blood samples have been archived for many years [17]. Samples were taken by venepuncture using EDTA as anticoagulant and were stored at -30°C until DNA extraction. We selected 72 samples from animals among which both sexes and a range of ages were represented (45 females aged 0–9 years and 27 male new-born calves).

Additionally, we used blood samples collected from a wild population of Soay sheep on the St Kilda archipelago in the Outer Hebrides (Scotland), which have been subject to individual-based monitoring and regular sampling since 1985 [18]. Blood samples were taken by venepuncture in August 2013, using heparin as an anticoagulant. Buffy coat fractions were prepared as follows: whole blood samples were centrifuged at 3,000 rpm for 10 minutes. The plasma layer was removed and remaining cells were washed by adding 0.9% NaCl solution. After centrifugation for 10 minutes at 3,000 rpm the intermediate buffy coat layer was collected, transferred to a 1.5 ml Eppendorf tube and stored at -20°C until further use. We selected samples from 48 different females aged 4–13 years for DNA extraction.

Ethics statement

Blood sampling from Holstein Friesian cattle and Soay sheep was approved by the Animal Experiments Committee (UK Home Office Project License Numbers: PPL 60/4278 and 60/3547, respectively).

DNA extraction

DNA from each cattle sample was extracted using the QIAGEN Genra Puregene kit (PG) based on a non-membrane salting out method and two silica membrane-based protocols of the

QIAGEN DNeasy Blood & Tissue kit: spin column (SC) and the 96-well plate (SP). DNA from each sheep sample was extracted using the PG and SC protocols.

According to the PG protocol, DNA is first isolated by removing red blood cells and lysing white blood cells. RNA and proteins are removed by enzyme digestion and salt precipitation, respectively. DNA is recovered by alcohol precipitation and dissolved in DNA hydration solution. The SC and SP protocols rely on a silica-based extraction method during which cells are lysed and transferred onto silica membranes to which DNA binds specifically during a centrifugation step. DNA is washed and finally eluted using a DNA hydration buffer. When possible, we performed different DNA extraction methods simultaneously on each sample. We followed the manufacturer’s protocol with certain alterations to improve yield and quality of DNA samples. The most important alternation was that the silica protocols were started with a red blood cell lysis step that allowed us after centrifugation to transfer only the white blood cell pellet dissolved in PBS onto the silica membranes. This step removed impurities in the beginning of the protocol and improved purity measurements greatly. SC samples were also prepared in duplicates that were run through the same silica membrane to improve DNA yield. All alternations are detailed in [S1 File](#). Fifteen cattle samples extracted by PG had to be re-purified following appendix C of the manufacturer’s manual.

Quality control of DNA extracts

We employed a strict quality control (QC) strategy during DNA extraction and qPCR to ensure that samples extracted by different methods were of similar quality, purity and integrity. Our aim was to minimize the risk of differences between DNA extraction measurements being due to sample quality rather than differences of methods themselves. Samples failing QC were excluded from our final analyses ([Table 1](#)).

We tested DNA yield and purity using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) with the software NanoDrop 2000. Samples with DNA concentrations less than 20 ng/μl were excluded from further investigation ([Table 1](#)). The average ratio of absorbance at 260 nm over 280 nm (OD 260/280) over two measurements was used to check for protein contamination and the average ratio at 260nm over 230nm (OD 260/230) was used to check for

Table 1. Number of samples after each quality control step by species and method of DNA extraction.

Quality control step	DNA extraction method				
	Cattle			Sheep	
	PG	SC	SP	PG	SC
1. Starting samples	72	72	72	47	47
2. DNA yield >20ng/ul on Nanodrop	66	71	66	47	47
3. Protein contamination (260:280 ratio > 1.7)	66	71	66	47	41
4. Salt contamination (260:230 ratio > 1.8)	61	71	66*	47	39
5. DNA yield >20ng/ul on Qubit/FLUOstar	61	71	62	47	39
6. DNA integrity score <3	61	69	56	47	36
7. Sample selection (samples passing all tests for all methods)	56	56	51	36	36
8. Number of RTL measurements (sample number x qPCR plates)	224	224	196**	144	144
9. qPCR efficiencies for each triplicate within 5% of mean plate efficiency	224	223	196	144	144
10. Triplicate sample Cq values had CV < 5%	223	221	196	142	144

PG = Genra Puregene Kit; SC = Spin Column protocol (DNeasy Blood & Tissue Kit); SP = Spin Plate protocol (DNeasy Blood & Tissue Kit)

* This step did not apply to SP.

**Four samples were run on two qPCR plates only, because they did not yield enough DNA for more measurements.

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salt contamination. Both proteins and some salts can act as qPCR inhibitors [19]. Extracts with $OD_{260/280} < 1.7$ or $OD_{260/230} < 1.8$ were excluded from further analyses for PG and SC methods. For SP, $OD_{260/230}$ readings were variable probably due to samples with low yields approaching the limit for accurate contaminant detection. We therefore decided not to exclude SP samples based on $OD_{260/230}$, although we applied the same $OD_{260/280}$ QC threshold as for the other methods. Note that results obtained from SP extracted samples behaved very similarly to the SC samples, despite the variable $OD_{260/230}$ ratios (see [Results](#)).

To assess DNA concentrations more accurately all PG and SC extracts were subsequently measured on a Qubit[®] 2.0 (Invitrogen) using a Qubit[®] dsDNA BR Assay kit (Invitrogen) according to the manufacturer's manual. SP extracts were measured on a FLUOstar Galaxy microplate reader (BMG LABTECH) using a Quant-iT[™] dsDNA Assay Kit (Invitrogen) following the manufacturer's instructions. Both procedures are based on the detection of a fluorophore that becomes fluorescent when bound to double stranded DNA. Measurements are evaluated in relation to standards with known DNA concentrations. Because the signal is specific for double stranded DNA (dsDNA) fluorescence spectroscopy measurements are more accurate for DNA yield than NanoDrop measurements. Samples with average concentrations lower than 20 ng/ μ l calculated over two measurements on either fluorometer were excluded from further investigation. DNA integrity was assessed visually by running 200ng on a 0.5% agarose gel with ethidium bromide at a final concentration of 0.8 μ g/ml. Gels were run at 100 mV and 200 mA for 45 minutes and then visualised with an AlphaImager TM 2200. Gels were visually scored for integrity on a scale of 1 to 5 ([Fig 1A](#)) and extracts with a score greater than 2 were removed from further analyses. DNA stock solutions were prepared by diluting extracts to a concentration of 10 ng/ μ l based on fluorescence measurements. PG extracts were diluted in DNA hydration solution (QIAGEN), and SC and SP extracts were diluted in buffer AE (QIAGEN).

Telomere length measurement

Leukocyte RTL was measured by qPCR [16] as the amount of telomeric DNA in a sample relative to the amount of a non-variable copy number reference gene. In order to identify the most appropriate reference gene we conducted preliminary analyses considering a variety of candidate reference gene primer pairs. The most consistent amplification profile and cleanest melting curve was obtained in both species using Primerdesign primers targeting the beta-2-microglobulin (*B2M*) gene (accession number: NM_001009284), which we selected as our reference gene. The selection of our reference gene was based on comparison of a panel of 12 candidate genes for sheep and 6 for cattle, supplied as part of the Primerdesign GeNorm kit (following Fairlie et al. 2016). *B2M* showed completely stable qPCR results indicative of non-variable copy number, and is well conserved and located on chromosome 10 of the bovine genome and chromosome 7 of the ovine genome [20,21]. For the telomere amplification, tel 1b (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT) and tel 2b (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT) primers were used [22]. Telomere primers were manufactured and purified with high performance liquid chromatography by Integrated DNA Technologies (IDT, Glasgow, UK).

The use of identical primers allowed us to use identical reaction conditions for both cattle and sheep qPCRs. We ran samples extracted by different methods and species on separate 384-well plates. Reactions for telomere and *B2M* primers were run in separate wells (monoplex qPCR) but on the same qPCR plate. Each qPCR plate was repeated four times over two days. Our calibrator sample came from a large volume of blood obtained from an individual cow or sheep, respectively. We extracted large quantities of DNA from each calibrator sample using different methods to match those applied to our experimental samples: PG, SC and SP for

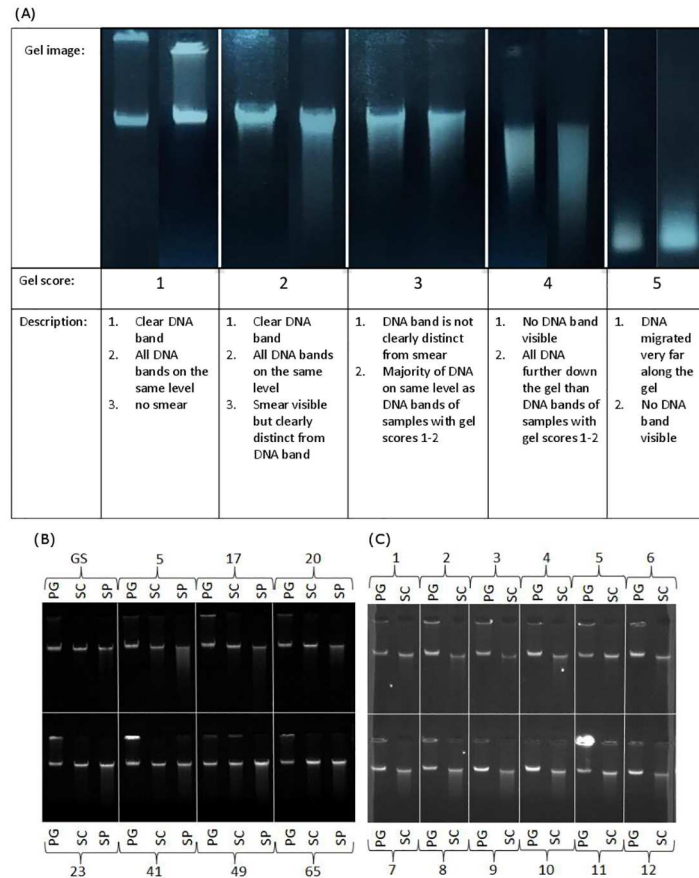


Fig 1. DNA integrity gels. (A) Illustrative DNA Integrity gels with gel scores. Example integrity gels for (B) Holstein Friesian cattle and (C) Soay sheep. Individual samples (represented by numbers in image) that were extracted with different DNA extraction protocols. (PG: Genra Puregene kit, SC: DNeasy spin columns, SP: DNeasy 96 well plate; GS: calibrator DNA (“golden sample”).

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cattle, PG and SC for sheep. In the cattle experiment, each qPCR plate included three calibrator samples, one for each of the extraction methods used (i.e., calibrator samples extracted with PG, SC and SP methods). In the sheep experiment, we only included the MS calibrator on each plate (i.e. PG-extracted calibrator on plates of PG-extracted samples and SC-extracted calibrator on plates of SC-extracted samples).

Samples and calibrators were loaded at a dilution of 1 ng/μl onto a 96 well plate (sample plate) that also contained a four step 1:4 serial dilution of calibrator DNA starting with 10 ng/μl as standard and nuclease free water as non-template control. A Freedom EVO 2150 robot (by TECAN) was used to transfer all samples, standards, calibrators and negative controls in triplicate onto a 384 well qPCR plate. The robot mixed 1 μl of the contents of the sample plate with 9 μl of master mix in each qPCR plate well. The master mix for both reactions contained 5 μl of LightCycler 480 SYBR Green I Master (Roche) per well. Telomere primers were used at a concentration of 900 nmol, *B2M* primers were used at 300 nmol. Nuclease-free water was added to the master mix to have a final volume of 10 μl per well.

The qPCR was performed on a LightCycler 480 (Roche) using the following protocol: Enzyme activation: 15 min at 95°C; then 50 cycles of: 15 s at 95°C (denaturation), 30s at 58°C (primer annealing), 30 s at 72°C (signal acquisition); melting curve: 1 min at 95°C, 30s at 58°C,

then continuous increase of temperature (0.11°C/s) to 95°C with continuous signal acquisition; Cool down: 10 s at 40°C. Melting curves showed a single peak with *B2M* primers rarely forming primer dimers in the negative controls. Telomere primers always form primer dimers due to the repetitive nature of their sequence. Evidence for primer dimer formation can be seen as melting peaks at slightly higher melting temperatures than the telomere qPCR product and also as amplification curves at very late cycles (average Cq for telomere negative controls: 38.1 (cattle) and 31.3 (sheep) compared to average Cq values of samples: 14.42 (SD = 0.76, cattle) and 13.52 (SD = 0.51, sheep)).

The software package LinRegPCR [23] was used to correct amplification curves for an estimated fluorescence baseline. The software also calculated well-specific amplification efficiencies. We used the mean efficiency across all wells on a plate, having excluded the upper and lower 5th percentiles, as our reaction efficiency for each amplicon group [23]. The mean qPCR efficiencies across plates calculated with LinRegPCR ranged between 93.1%-94.2% (cattle) and 93.5%-94.0% (sheep) for the *B2M* reaction, and 93.6%-94.4% (cattle) and 92.5%-95.5% (sheep) for the telomere reaction. We set a constant fluorescence threshold within the window of linearity across all plates for the calculation of Cq values. The threshold was for *B2M* 0.221 in cattle and 0.1 in sheep and for the telomere amplification 0.256 and 0.1 in cattle and sheep, respectively.

We calculated mean qPCR efficiencies separately for both amplicon groups (*B2M* and telomere reaction) for each qPCR plate using LinRegPCR. Samples were excluded from final analysis if at least one of their triplicate amplifications had a qPCR efficiency that was 5% higher or lower than the mean efficiency for the respective amplicon. Also, samples were excluded if their Cq values had a coefficient of variation (CV) > 5% across triplicates. Elimination of samples that failed quality control for qPCR efficiency or Cq values ensured high intra-plate repeatabilities and efficiencies, although less than 1% of our samples were excluded based on these criteria (see Table 1).

RTL was calculated using following formula described by Pfaffl [24]:

$$RTL = \frac{E_{TEL}^{Cq_{TEL(Calibrator)} - Cq_{TEL(Sample)}}}{E_{B2M}^{Cq_{B2M(Calibrator)} - Cq_{B2M(Sample)}}$$

where E_{TEL} and E_{B2M} are the reaction efficiencies for the plate for the respective amplicon group calculated by LinRegPCR; $Cq_{TEL[Calibrator]}$ and $Cq_{B2M[Calibrator]}$ are the mean Cq values across triplicates for the telomere and *B2M* reactions, respectively, for the plate's calibrator sample; and $Cq_{TEL[Sample]}$ and $Cq_{B2M[Sample]}$ are the mean Cq values across triplicates for telomere and *B2M* reactions, respectively, for the focal cattle or sheep sample.

An aim of our study was to test whether the use of a MS calibrator could control for differences in RTL amongst extraction methods. Therefore, in our initial cattle experiment we calculated RTL with the equation above but using three different calibrators: (1) a MS calibrator, (2) a calibrator extracted with a single method across all plates, arbitrarily choosing PG (termed "PG calibrator"), and (3) a constant Cq value across all plates ("no calibrator"). We chose constants of 26 for the reference gene and 14 for telomeres, as these were the average sample Cqs for these amplicons in our cattle experiment. The use of a constant Cq in the above equations allowed us to examine how well the use of a plate-specific calibrator (either MS or PG calibrators) accounted for plate-to-plate variation in RTL measures, whilst keeping RTL values on a similar scale as the RTLs calculated with MS and PG calibrators. In the subsequent sheep experiment, we only compared the MS calibrator with the no calibrator calculations (25.99 for reference gene and 13.71 for telomeres). We also examined variation in the raw Cq values for the telomere and *B2M* reactions. It is important to note that higher Cq values represent lower concentrations of telomere or reference gene and vice-versa in our RTL calculations.

Statistical Analysis

Each sample was run on four identical qPCR plates per DNA extraction method. We calculated the Pearson's correlation coefficient for the individual RTL measurements between all possible plate combinations. We took the average RTL for a sample across the four plates within each extraction method and calculated the Pearson's correlation coefficient among methods. We calculated the CV—i.e. the standard deviation divided by the mean—across replicates of each sample both across all plates and within plate using the same extraction method. Pooled CVs across samples were calculated as the geometric mean CV.

Linear mixed models were used to estimate the repeatability of RTL measurements and Cq values for a given sample, the degree of plate to plate variation, and the effect of DNA extraction method on mean RTL. The model of analysis included the random effects of sample, sample-by-extraction method interaction and plate, and the fixed effect of DNA extraction method. Variance components for the random effects were estimated using restricted maximum likelihood. The sum all variance components constituted the total phenotypic variance. The repeatability of sample RTL across plates and methods was calculated as the ratio of the sample variance to the total phenotypic variance. The ratio of the sample-by-extraction method interaction to total phenotypic variance provided an estimate of the proportion of variance attributable to differences in RTL among extraction methods within a sample, whereas the ratio of the plate effect to total phenotypic variance expressed the proportion of variance attributable to differences in the mean RTL among plates. We tested the significance of any differences in mean RTL associated with DNA extraction method by comparing models with and without extraction method as a fixed effect using a likelihood ratio test. We ran separate models for RTL calculated using MS calibrators (both species), PG calibrators (cattle only) and no calibrator (both species). We made the same comparisons for the reference gene and telomere Cq values in both species. All statistical analyses were performed in R Studio with R 3.1.2 [25] with mixed-effects models being implemented using the 'lme4' library.

Results

DNA yield and integrity with different DNA extraction methods

A total of 56 of our PG and SC cattle samples, 51 of our SP cattle samples, and 36 of our sheep samples passed all quality controls for all DNA extraction methods and were used for RTL measurement (resulting in RTL measurements for a total of 235 DNA samples; Table 1). DNA yield was method dependent. The non-silica membrane-based PG extraction kit yielded the highest DNA concentrations (cattle: mean = 341 ± 6 ng/ μ l; sheep: mean = 282.6 ± 2 ng/ μ l) and highest total yields of DNA (cattle: mean = 76 ± 2 μ g; sheep: mean = 74 ± 1 μ g). The SC method produced substantially lower yields (cattle: mean concentration = 120 ± 2 ng/ μ l, mean total yield = 12 ± 0.2 μ g; sheep: mean concentration = 68 ± 1 ng/ μ l, mean total yield = 15 ± 0.2 μ g) and the SP method lower still (cattle: mean concentration = 38 ± 0.6 ng/ μ l; mean total yield = 3 ± 0.05 μ g). However, initial whole blood volumes of cattle varied between DNA extraction methods (PG: 3 ml, SC: 600 μ l, SP: 300 μ l), whereas the same volumes of sheep buffy coat were used in all cases.

We also noticed that DNA integrity gels varied in appearance across extraction methods (Fig 1B). PG extracts showed the cleanest bands with no signs of smears and thus no signs of DNA disintegration. Based on our numeric integrity gel score (Fig 1A) all PG samples for both species scored a 1 (best score) while all spin column samples for sheep and 2 out of 69 samples for cattle scored a 2. Of the SP samples the majority of samples (83.9%) passed with a gel score of 2. A total of 11 SC or SP extracts from both species failed quality control based on their integrity gel score (Table 1).

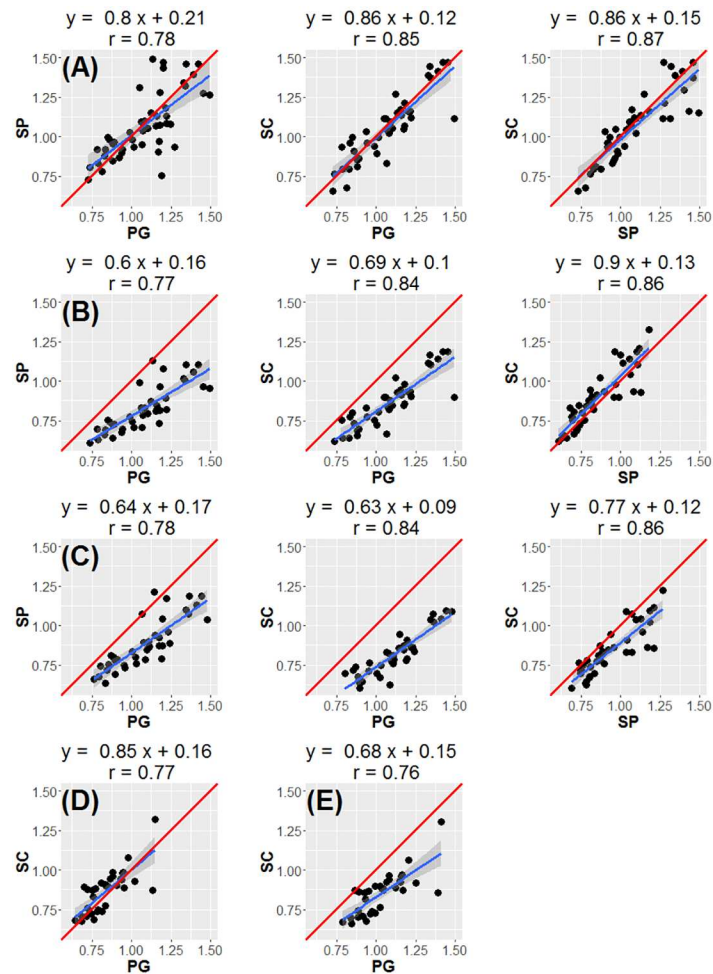


Fig 2. Correlations between methods. Correlations between RTL measurements from different DNA extraction methods (PG: Gentra Puregene kit; SC: DNeasy spin columns; SP: DNeasy 96 well plate): Cattle, method-specific calibrator (A); Cattle, Puregene calibrator (B); Cattle, no calibrator (C); Sheep, method-specific calibrator (D); Sheep, no calibrator (E). Regression lines and their 95% confidence interval are shown in blue and grey, respectively, with red lines reflecting a hypothetically perfect correspondence (slope of one, intercept of zero).

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Repeatability of telomere length measurements & effects of DNA extraction method

We found relatively high correlation coefficients and low CVs across plates for RTL measurements of the same sample in both species. All correlation estimates both within DNA extraction method (across plates) and between methods for the two species are summarized in [S2 File](#). Correlations among RTL measurements from the same sample, calculated using a MS calibrator, among plates ranged from 0.87 to 0.96 for cattle, and 0.83 to 0.93 for sheep ([S2 File](#)). Correlations between average RTL measurements derived from different extraction methods and using different calibrators are summarised in [Fig 2](#). Using a MS calibrator, correlations between the PG and SC methods were 0.85 for cattle and 0.77 for sheep, whilst in cattle the correlation between PG and SP was 0.78 and between SC and SP 0.87 ([Fig 2](#)). The correlation coefficients were comparable when a PG calibrator or no calibrator was used for RTL calculation ([Fig 2](#)). However, when fitting regression lines among samples extracted using different

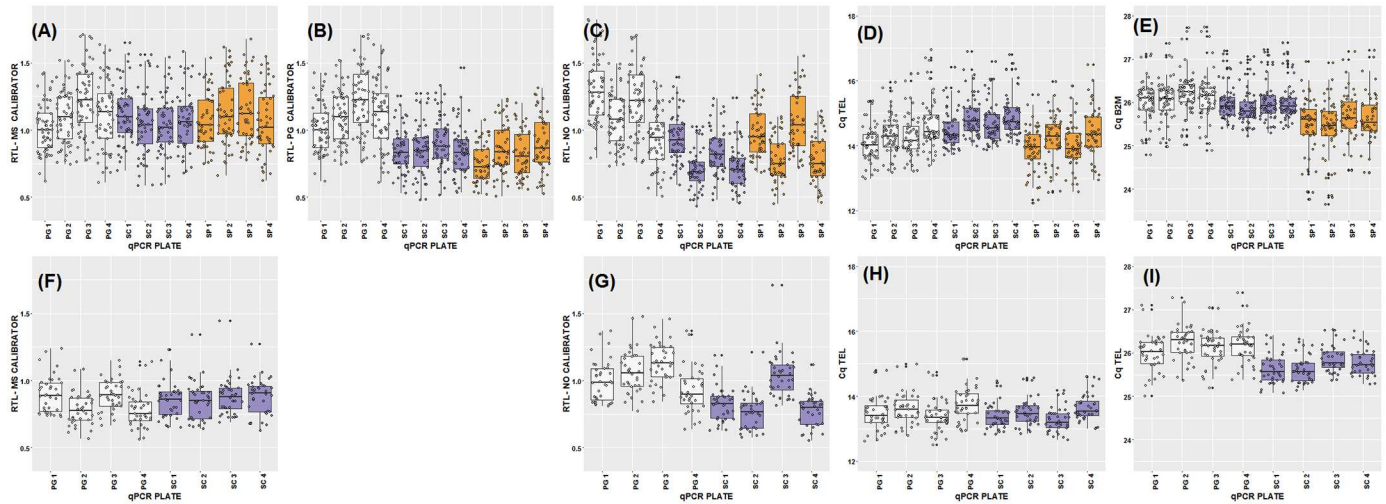


Fig 3. Raw RTL and Cq values. RTL or Cq values by DNA extraction method and qPCR plate for cattle (A-E) and sheep (F-I). RTL calculated with method specific (MS) calibrator (A + F), Puregene (PG) calibrator (B), no calibrator (C+G). Cq values for telomere reaction (D+H) and control gene *B2M* (E+I). Colours represent DNA extraction methods. White: Gentra Puregene, blue: DNeasy spin columns, orange: DNeasy 96 well plate.

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methods, application of the MS calibrator clearly produces regression slopes much closer to one with intercepts close to the origin (Fig 2). The average CV across all plates was 8.2% in cattle (12 plates, 3 methods), and 8.1% in sheep (8 plates, 2 methods). Within extraction method, CVs across plates were 9.2% and 8.2% for PG, 5.1% and 4.5% for SC, for cattle and sheep, respectively, and 5.2% for the SP in cattle only.

In both cattle and sheep, we found significantly ($P < 0.05$) higher mean RTL in samples extracted using the non-membrane-based method (PG) compared to those extracted with the silica membrane-based methods (SC and SP), when using either the PG calibrator or no calibrator in calculations (Fig 3, Table 2). This reflects genuine underlying differences in the average TL among DNA extracted from the same sample by different methods, as has been reported elsewhere [10,11]. These differences are underpinned by either or both lower telomeric Cq and higher reference gene Cq values in the PG extracted samples compared to the other methods (Fig 3D, 3E, 3H & 3I). In both species, there was notable variation in the telomeric Cq values across plates run on the same day, with the first plate having lower values than the second (Fig 3D & 3H). As would be expected, application of a plate-specific calibrator (either PG or MS calibrators) removed the within-day variation in RTL and substantially reduced among-plate variation (Table 2; Fig 3). Importantly, the differences in mean RTL among extraction methods became non-significant and sample repeatabilities were increased when a MS calibrator was used to calculate RTL (Fig 3, Table 2). This shows that using a MS calibrator to calculate RTL can account for observed effects of DNA extraction method on the underlying Cq values (Table 2; Fig 3).

Discussion

In the present study, we addressed the effect of DNA extraction method on RTL measurements by comparing two silica membrane-based kits (SC and SP) with a kit that uses a non-membrane-based salting out method (PG). As expected [26], we found that the salting out method produced higher DNA yields and that silica membrane-based methods were associated with some observable loss of DNA integrity (Fig 1). A number of studies using human blood samples report significant differences in mean RTL depending on the DNA extraction method

Table 2. Variance component and parameter estimates.

	σ^2_{Total}	σ^2_{Sample}	$\sigma^2_{SamplexMethod}$	σ^2_{Plate}	$\sigma^2_{Residual}$	r^2_{Sample}	$r^2_{SamplexMethod}$	r^2_{Plate}	X^2_{Method}	P Method	Effect PG vs. SC \pm SE	Effect PG vs. SP \pm SE
Cattle												
RTL calculated with MS calibrator	0.053	0.038	0.007	0.003	0.005	0.717	0.130	0.064	0.710	0.701	-0.035 \pm 0.045	-0.016 \pm 0.045
RTL calculated with PG calibrator	0.041	0.028	0.006	0.004	0.004	0.671	0.137	0.098	20.434	<0.001	-0.244 \pm 0.048	-0.283 \pm 0.048
RTL calculated with no calibrator	0.057	0.028	0.006	0.020	0.004	0.482	0.103	0.354	9.537	0.008	-0.330 \pm 0.102	-0.237 \pm 0.102
Cq for B2M (reference gene)	0.293	0.126	0.155	0.004	0.008	0.430	0.528	0.015	31.069	<0.001	-0.138 \pm 0.089	-0.638 \pm 0.091
Cq for telomere amplification	0.507	0.296	0.140	0.054	0.017	0.585	0.276	0.106	11.407	0.003	0.409 \pm 0.179	-0.271 \pm 0.180
Sheep												
RTL calculated with MS calibrator	0.020	0.012	0.003	0.002	0.002	0.615	0.162	0.113	0.620	0.431	0.027 \pm 0.036	/
RTL calculated with no calibrator	0.034	0.014	0.004	0.012	0.003	0.430	0.113	0.359	4.815	0.028	-0.179 \pm 0.079	/
Cq for B2M (reference gene)	0.150	0.051	0.083	0.013	0.003	0.338	0.555	0.084	11.185	0.001	-0.414 \pm 0.105	/
Cq for telomere amplification	0.238	0.108	0.099	0.026	0.006	0.452	0.417	0.108	0.498	0.481	-0.091 \pm 0.136	/

MS calibrator: Method specific calibrator, PG calibrator: Puregene extracted calibrator

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used [10–14]. We found that silica membrane-based DNA extraction methods produced shorter RTL measurements on average than the salting out method in both cattle and sheep. This is consistent with two previous studies in humans, which argued that silica membrane based DNA extraction methods reduce average RTL [10,11]. However, the physical and biochemical causes of these observed extraction method effects on RTL measurements are currently unknown, and determining these causes is an important next step for research in this area.

We found that the rank order of RTL measurements among samples is largely unaffected by DNA extraction methods. Across extraction methods, our RTL measures showed reasonably high repeatabilities and inter-plate correlations and low inter-plate CVs that were close to those reported in the qPCR telomere literature [7,14,16]. The aforementioned studies on human samples do not offer clear insight into how extraction methods affect the rank order of RTL measurements. One study reported relatively high correlations among samples extracted by QIAamp spin columns (QIAGEN) versus a magnetic bead extraction (Spearman's $\rho = 0.71$) [11]; another study found a moderate correlation between a magnetic bead and a salting out extraction (Pearson's $r = 0.54$) [14]. A third study found very low and not statistically significant correlations ($r < 0.21$) [12], and two of the studies did not present among sample correlations [10,13]. The absence of a strong correlation among RTL measurements based on different DNA extraction methods is a profoundly alarming result for research on telomere dynamics. If rank order of RTL is generally altered by underlying aspects of sample preservation, then associations among RTL and environmental, genetic and health measures within studies could themselves depend on the extraction method used. However, the one study reporting low correlations among RTLs based on different extraction method used DNA samples that would have failed our QC criteria [12] and it seems likely that the low correlations may be the result of variation in the level of DNA impurities that might have acted as qPCR inhibitors. Our results show that, as long as rigorous QC criteria are applied throughout telomere measurement protocols, the rank order of samples is very largely preserved regardless of the DNA extraction method used, despite the distribution of RTL estimates changing (Fig 2). Failure to carefully monitor and control the integrity and purity of DNA is likely to result in increased sampling error which will reduce the repeatability of results both within and among studies of telomere dynamics.

Importantly, our results show that it is possible to account for differences in mean RTL associated with DNA extraction method using a DNA extraction method-specific calibrator. Our reading of the literature suggests it is unusual for qPCR-based telomere studies in both epidemiology and ecology to provide much information about the source or preparation of the calibrator sample used. The five previous studies of DNA extraction method effects on RTL discussed above presumably used a calibrator sample extracted using only one extraction method, although most of them fail to explicitly state what kind of calibrator was used [10–13] and how it was extracted [10–14]. This is entirely reasonable given the aim was to test for differences in the telomere to control gene ratios associated with DNA extraction method. In this study, we have demonstrated a relatively simple approach that could account for DNA extraction method effects on RTL that could potentially allow researchers to perform qPCR based telomere studies combining samples extracted in different ways. By extracting large quantities of DNA from a single large sample of blood by different methodologies and running these on appropriate plates, we were able to apply an extraction method-specific calibrator in our calculations of RTL. This accounted for the extraction method effects on mean RTL which were observed in our two data sets when the standard calibration approach was used. More generally, our data suggest that within qPCR-based studies of TL, calibrator samples could be used for more than just accounting for plate to plate variation. As long as DNA integrity and purity

is carefully controlled, calibrator samples derived from the same original sample but collected, stored or extracted in different ways could conceivably be used to control for systematic effects of variation in sample preparation on RTL.

It is obviously preferable to use a completely consistent approach and extract DNA using the same method within a study. However, a major challenge in the study of telomere dynamics is to generate sufficiently detailed longitudinal data to determine whether variation in TL observed later in life is the result of differences set in early life or differences in attrition rates across life [27]. Addressing this challenge in long-lived animals will inevitably require the use of long-term longitudinal archived samples, in which samples may have been stored or DNA extracted in different ways over time. Our calibrator-based approach could allow such valuable longitudinal samples to be compared within a single study, but it would need to be carefully validated each time it was applied. We would advocate applying similarly stringent quality control on DNA integrity and purity as here, even though this may reduce available sample size. Before applying a method-specific calibrator approach to archived samples prepared in different ways, it would also be crucial to run a similar experiment to establish the repeatability of RTL measures among samples that have been experimentally exposed to the relevant differences in sample collection, storage or DNA extraction.

Conclusion

This study adds to the emerging literature showing that DNA extraction methods may affect the mean RTL measurement produced by qPCR techniques. We present the first evidence for such effects in non-human vertebrates, documenting similar results in two ruminant species of considerable economic and agricultural importance in which TL variation has recently been examined with some exciting initial results [28–30]. We also show that RTL measurements derived from different DNA extraction methods are highly correlated when rigorous DNA quality control is applied. Our results also suggest that the application of method-specific calibration in qPCR studies of RTL could allow researchers to effectively use valuable historical archives of samples that have been prepared or extracted in different ways, accounting for effects of methodological variation on mean RTL.

Supporting Information

S1 File. Modified DNA extraction protocols.
(DOCX)

S2 File. Correlations within and between DNA extraction methods.
(DOCX)

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Visualization: LS.

Writing – original draft: LS DN.

Writing – review & editing: SU JF AP JI MC GB.

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RESEARCH ARTICLE

Longitudinal changes in telomere length and associated genetic parameters in dairy cattle analysed using random regression models

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Abstract

Telomeres cap the ends of linear chromosomes and shorten with age in many organisms. In humans short telomeres have been linked to morbidity and mortality. With the accumulation of longitudinal datasets the focus shifts from investigating telomere length (TL) to exploring TL change within individuals over time. Some studies indicate that the speed of telomere attrition is predictive of future disease. The objectives of the present study were to 1) characterize the change in bovine relative leukocyte TL (RLTL) across the lifetime in Holstein Friesian dairy cattle, 2) estimate genetic parameters of RLTL over time and 3) investigate the association of differences in individual RLTL profiles with productive lifespan. RLTL measurements were analysed using Legendre polynomials in a random regression model to describe TL profiles and genetic variance over age. The analyses were based on 1,328 repeated RLTL measurements of 308 female Holstein Friesian dairy cattle. A quadratic Legendre polynomial was fitted to the fixed effect of age in months and to the random effect of the animal identity. Changes in RLTL, heritability and within-trait genetic correlation along the age trajectory were calculated and illustrated. At a population level, the relationship between RLTL and age was described by a positive quadratic function. Individuals varied significantly regarding the direction and amount of RLTL change over life. The heritability of RLTL ranged from 0.36 to 0.47 (SE = 0.05–0.08) and remained statistically unchanged over time. The genetic correlation of RLTL at birth with measurements later in life decreased with the time interval between samplings from near unity to 0.69, indicating that TL later in life might be regulated by different genes than TL early in life. Even though animals differed in their RLTL profiles significantly, those differences were not correlated with productive lifespan ($p = 0.954$).

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Introduction

Telomeres are located at the ends of linear chromosomes. They consist of non-coding nucleotide tandem repeats (TTAGGG in vertebrates) and attached proteins of the shelterin complex [1–3]. Since telomeres were first shown to shorten with the number of cell divisions *in vitro* [4], they have been intensely studied in relation to ageing and lifespan in various species *in vivo* [5–9]. Such studies have reported mixed results. While some observed a positive correlation between telomere length and longevity [5,10–12], others found no relationship [13,14]. Many authors claimed that longitudinal studies were necessary to better understand telomere dynamics within the individual, and to investigate the association of not only telomere length but also change in telomere length with lifespan [10,15–17]. In longitudinal studies of Alpine swifts and Seychelles warblers, faster telomere attrition, but not telomere length *per se*, was associated with poorer survival [18,19]. In humans telomere length maintenance was associated with better survival than telomere length attrition in patients with cardiovascular disease [20,21]. However, the relationship between telomere length attrition and survival has not been investigated in a livestock species to date.

Genetic studies on telomere length are rare outside the human literature. In humans it has been shown that telomere length is a quantitative trait that is controlled by many different loci [22–26]. Heritability estimates are available for humans, sand lizards and kakapos and range from 0.39 to 0.82 in those species [27–33]. Outside those studies heritability estimates are missing from the literature. It has been shown in the above mentioned species that telomere length is a heritable trait, but it is unclear if heritability estimates change over life or are relatively constant. A changing impact of environmental effects on telomere length might change heritability estimates over time. For animal breeders it is interesting to know which proportion of a trait at any time is caused by genetic effects and therefore possible to influence with breeding.

In the livestock sector there is a growing interest in using telomere length as a biomarker for health, productive lifespan and animal welfare [34,35]. However, longitudinal studies that investigate change in telomere length within individuals are largely missing from the livestock literature. In the present study we are interested in the rate and direction of telomere length change and the relationship of different telomere length change profiles with productive lifespan. We use random regression models which were initially developed to describe lactation curves in dairy cattle [36,37] for the analysis of telomere length profiles. They allow the fitting of an overall fixed curve across time which describes the population trend, and individual random animal curves (profiles) as deviations from the former. Random regression models take into account the correlation among repeated measurements within an individual, which is usually greater than the correlation of measurements between animals [38]. Over the last two decades random regression models have been applied to many studies in genetics and evolutionary ecology addressing the change of a broad range of traits over time. Examples of studied traits in genetics include milk yield [39], milk fat and protein content [40], somatic cell count [41], body condition score [42,43], body energy [44] and carcass traits [45]. In evolutionary ecology studied traits included fitness [46], body size [47], body weight in relation to faecal egg counts [48] and antler size [49]. To our knowledge, only a single study has used random regression models for the analysis of longitudinal telomere data so far [18]. However, the study was based on a rather small dataset (373 samples of 204 individuals; more than half of the individuals were sampled once only) and could not find a statistically significant difference in telomere length profiles.

The objectives of the present study were to 1) characterize the change in bovine relative leukocyte telomere length (RLTL) across the lifetime in Holstein Friesian dairy cattle, 2) estimate genetic parameters of RLTL over time and 3) investigate the association of differences in individual RLTL profiles with productive lifespan.

Materials and methods

Ethics statement

Blood sampling of Holstein Friesian cattle was approved by the Animal Experiments Committee (UK Home Office Project License Number: PPL 60/4278).

Data

Animals used in this study were Holstein Friesian dairy cattle of the Langhill herd that were kept at the Crichton Royal Research Farm in Dumfries (Scotland, UK). All animals in this herd belong to one of two distinct genetic lines (selected for high milk fat and protein yield vs. control). Furthermore, cows are randomly allocated to two different diets that contain either a high or low proportion of forage. These genetic lines and diets were set up over 30 years ago to accommodate genetic and nutritional scientific studies [50].

We measured RLTL in 1,328 longitudinal samples of 308 female animals born between 2008 and 2014. Animals were approximately equally split between genetic lines and diets. All animals were blood sampled once at birth and then at least once more during their lifetime. On average, 4.3 samples were taken per animal. At the end of the study 244 out of 308 animals were dead and had recorded productive lifespan measurements. Productive lifespan was defined as the time between the animal's birth and culling in days. Productive lifespan differs from longevity measurements in humans and natural populations, because dairy cattle rarely die of natural causes. However, we argue that productive lifespan is still biologically meaningful, because animals are not culled randomly but usually for fertility or health reasons.

DNA was extracted from whole blood samples using DNeasy spin columns (QIAGEN) and each sample had to pass internal quality control steps which were 1) yield and purity measured on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) had to fulfil the minimum requirements of: yield > 20 ng/μl, 260/280 ratio > 1.7 and 260/230 ratio > 1.8 and 2) integrity gel scores had to be between 1–2 [51]. RLTL was measured by qPCR using 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') primers [52] for the telomere amplification and beta-2-microglobulin (B2M) primers (Primerdesign, accession code NM_001009284) for the reference gene amplification [51]. An identical sample—the so-called calibrator or golden sample—was repeated on every plate to correct for measurement error that is associated with the qPCR plate. The number of cycles at which the qPCR amplification curve crosses a set fluorescence threshold (the Cq value) was determined for each sample for telomere and B2M reactions. Raw Cq measurements were baseline corrected using the software LinReg PCR [53]. The same software was used to calculate the reaction specific qPCR efficiencies E_{TEL} and E_{B2M} that were in turn used in following formula [54] to calculate RLTL:

$$RLTL = \frac{E_{TEL}^{Cq_{TEL(Calibrator)} - Cq_{TEL(Sample)}}}{E_{B2M}^{Cq_{B2M(Calibrator)} - Cq_{B2M(Sample)}}} \quad (1)$$

The Cq values corresponding with the calibrator sample were $Cq_{TEL(Calibrator)}$ and $Cq_{B2M(Calibrator)}$ for the telomere and the B2M reaction respectively. Cq values of the individual samples were $Cq_{TEL(Sample)}$ and $Cq_{B2M(Sample)}$.

Individual samples were measured on 25 qPCR plates in total which had 8 rows for each reaction. RLTL data were logarithmically transformed to achieve normal distribution (Shapiro-Wilk normality test: $W = 0.9985$, $p = 0.299$). Because of the increasing scarcity of data points after the age of 60 months, this age was used as the cut-off for data visualisation. The

pedigree included 11,003 animals spread over 27 generations. The animals with RLTL measurements were descendants of 40 sires and 241 dams.

Data analysis

The following random regression model was used for the analysis of longitudinal RLTL data:

$$Y_{ijk} = \text{BirthYear}_j + \text{GeneticGroup}_j + q\text{PCRplate}_{ij} + q\text{PCRrow}_{ij} + \sum_{k=0}^n P_{jkt} b_k + \sum_{k=0}^n P_{jkt} u_{jk} + e_{ijk} \quad (2)$$

where Y_{ijk} = the i^{th} RLTL measurement for animal j using a Legendre polynomial of the order k . BirthYear_j represents the fixed effect of the year in which animal j was born; GeneticGroup_j stands for the fixed effect of the genetic group of animal j ; $q\text{PCRplate}_{ij}$ and $q\text{PCRrow}_{ij}$ of a particular sample i of animal j was included as fixed effects ($q\text{PCRplate}_{ij}$ and $q\text{PCRrow}_{ij}$); fixed effects regression coefficients are represented by b_k , while u_{jk} stands for the k^{th} order random regression coefficients for the additive genetic effects of animal j ; P_{jkt} represents the k^{th} order of Legendre polynomial fitted to the measurement i of animal j at the age t in months; the random residual variance is e_{ijk} . Sampling intervals and age at sampling (after the initial record) differed among individuals.

Model (2) included fixed effects that remained statistically significant ($p < 0.05$) after backwards eliminating all tested non-significant effects (such as birth season, birth weight, weight at sampling, body condition score, and feed group) and the genetic group of the animal. The fixed and random regressions, both modelled with polynomial functions, described the average RTL change across age, and individual animal deviations from the average, respectively. The latter pertained to the animal's additive genetic effect. The animal's permanent environment was also examined as a random factor but had a negligible effect (see [S1 File](#)).

We tested if the residual variance of different age groups differed significantly implying a heterogeneous variance structure. We first considered four different age groups (0–12 months, 13–24 months, 25–40 months and older than 40 months) and then two different age groups (younger and older than 2 months) but did not find a significant difference in residual variance between any age groups (see [S1 File](#)). Therefore, a homogeneous residual variance structure was assumed for the subsequent analysis.

The Akaike information criterion (AIC) was used to assess 1) if the introduction of the random animal genetic effect improved the model fit compared to a model that only included fixed effects; this would suggest that animals differ in their intercept (average RLTL across all measurements); 2) if Legendre polynomials fitted to the random animal genetic effect improved the model fit further, thereby suggesting that animals also differ in their slope (RLTL dynamics). A difference of two units in AIC corresponds to an approximate significance of $p < 0.05$. Within the range of two units the simpler model was preferred over the more complicated [55–57]. In the end, quadratic polynomials were fitted to both the overall fixed curve and the individual random animal deviation.

All statistical analyses were conducted with the ASReml software version 4.1 [58].

Calculation of the fixed and random curves. The fixed curve that illustrates RLTL dynamics at a population level was calculated as the sum of the products of the Legendre polynomial order residuals for a given age and the corresponding fixed regression coefficients. This was repeated across all ages in the trajectory. Random regression models allow the calculation of an individual profile of RLTL change over age for each animal as a deviation from the population mean (fixed curve). The model output provides estimates (solutions) for each animal and each order of polynomial fitted in the model. The random curves were calculated

simply by summing solutions for each animal and test month across all products of the n^{th} order polynomial with the n^{th} order polynomial residual. The standard error was calculated in parallel by using the standard errors associated with the solutions for the same calculation. Eigenvalues were calculated to estimate the amount of variance between animals that is due to 1) the intercept and 2) the shape of individual curves. Eigenfunctions were calculated to analyse the direction of each effect.

Variance components and genetic parameters. The additive genetic variance (V_A) for each month was calculated using following formula [38]:

$$V_A = pKp' \tag{3}$$

Where p is a $1 \times k$ vector (k is the order of the fitted Legendre polynomial) containing the residuals for each polynomial order for the given month, K is a matrix containing the REML estimates of (co)variance components and p' is the transposed p vector. The heritability of RLTL and its standard error were calculated at birth and for each consecutive month. Also, the genetic correlations of RLTL at birth with each following month were calculated. Detailed information about those calculations can be found in [S1 File](#).

Analysis of the association of RLTL dynamics with productive lifespan. Out of 308 animals 244 were dead by the end of the study and produced exact productive lifespan measurements. To investigate if different RLTL profiles were associated with a difference in productive lifespan, individual RLTL random curves (profiles) were clustered using the R library `kmlShape`

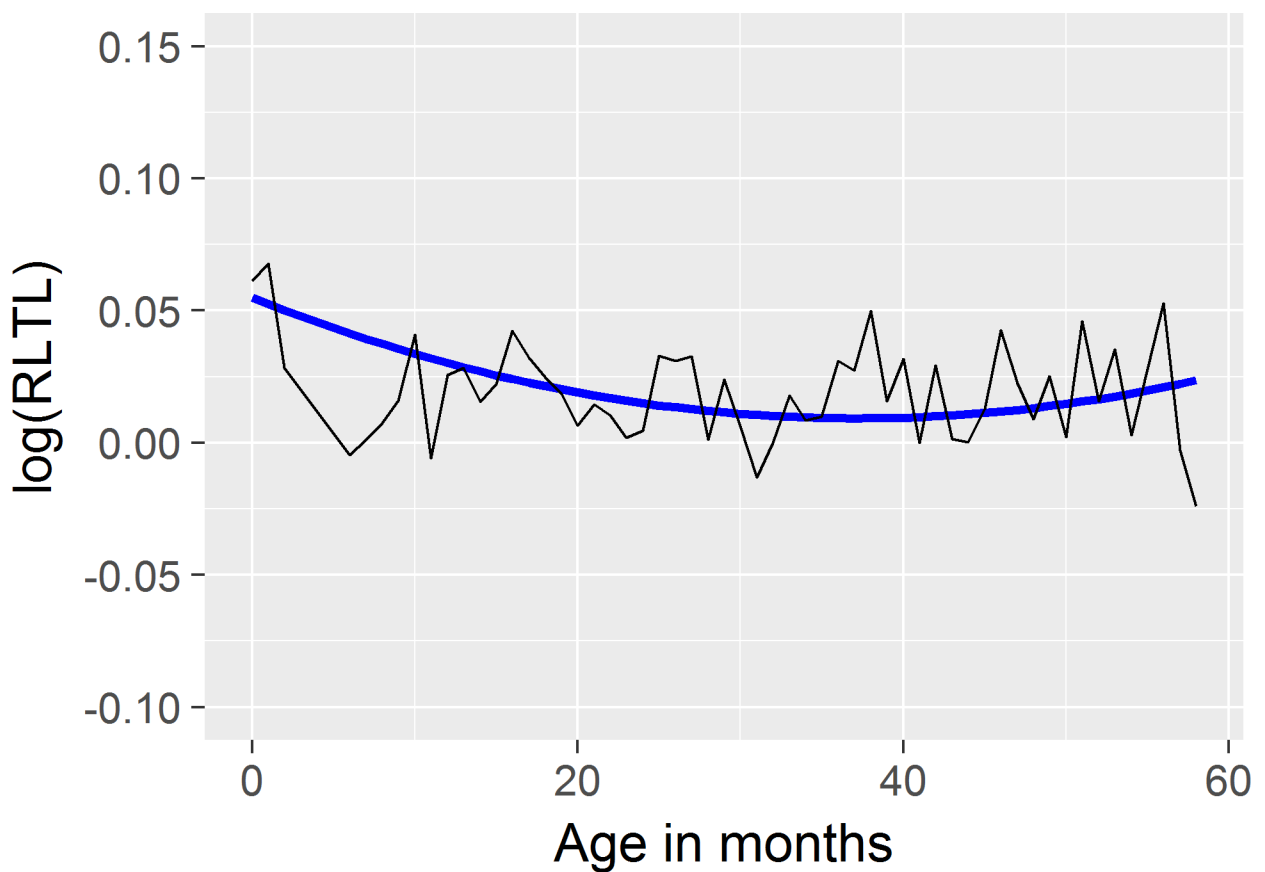


Fig 1. Fixed curve of logarithmically transformed relative leukocyte telomere (RLTL) data. Blue line: quadratic Legendre polynomial function of age; black solid line: phenotypic RLTL measurements for each month.

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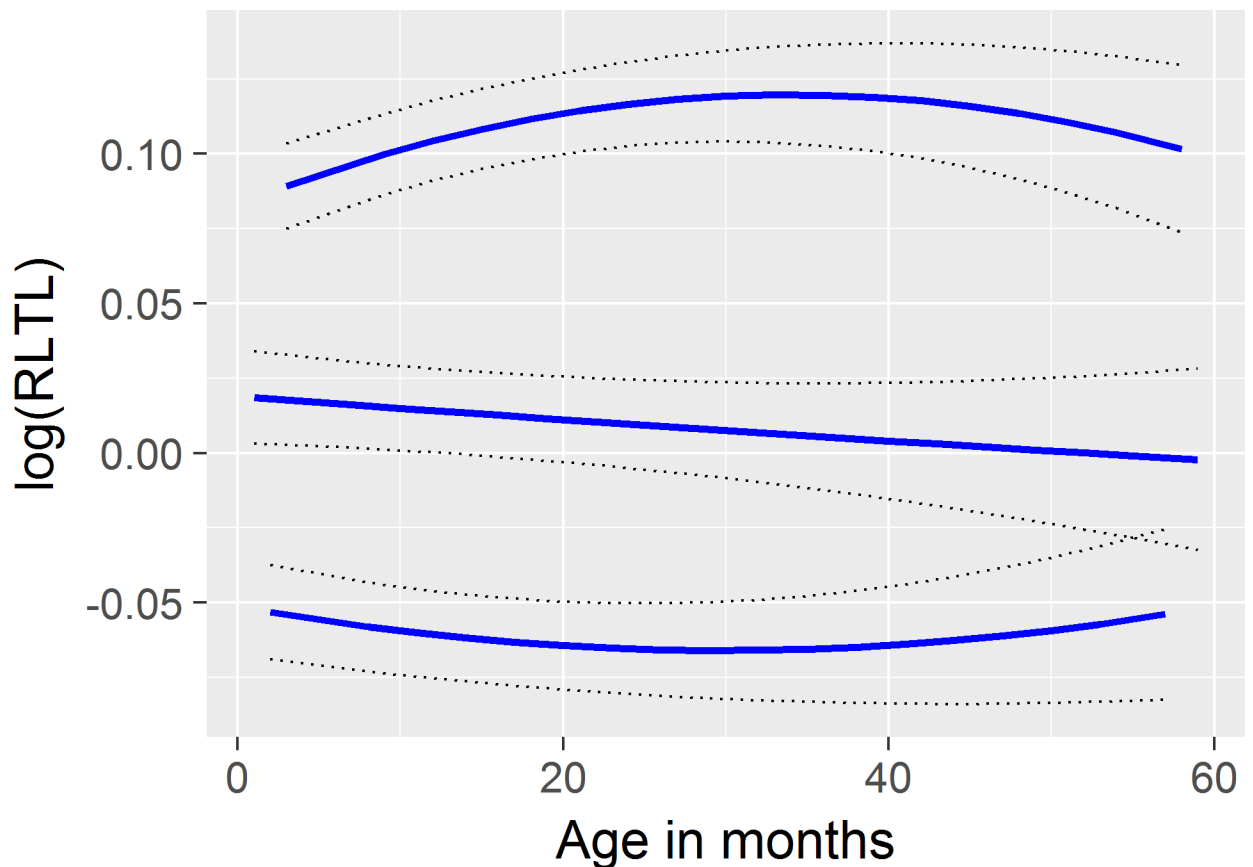


Fig 2. Examples for three individual animal RLTL curves (blue lines) with standard error (black, dotted lines), expressed as deviation from the fixed curve. Animals were chosen randomly to illustrate the variability between individual curves.

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[59] in five groups. We decided for five clusters to explore a difference in animals that maintain their RLTL in contrast to those who early in life either mildly or moderately shorten or elongate their RLTL, respectively. The association between productive lifespan and RLTL cluster was investigated with a Cox proportional hazard analysis. This analysis allows fitting maximal known survival times as right-censored data to account for animals that are still alive. For living animals age in days at the first day of the present year was used for the calculation of the maximal known survival time. A Wald test was used to determine the significance of the relationship between RLTL profiles and productive lifespan.

Results

Raw RLTL measures ranged from 0.693 to 1.727 with a mean of 1.082. The coefficient of variation was 0.162. The model that included the animal identity as a random effect fitted the data significantly better than a model including only the fixed effects (delta AIC = 204.97) suggesting that animals differed significantly in their average RLTL across time. Fitting animal identity with pedigree information further improved the model fit (delta AIC = 55.46). Fitting an individual curve for each animal (using a quadratic Legendre polynomial) additionally increased the model fit (delta AIC = 3.24), meaning that monthly RLTL dynamics also differ among individual animals. A quadratic Legendre polynomial fitted marginally better than a linear function (delta AIC = 2.07) and had the advantage that the same order of Legendre

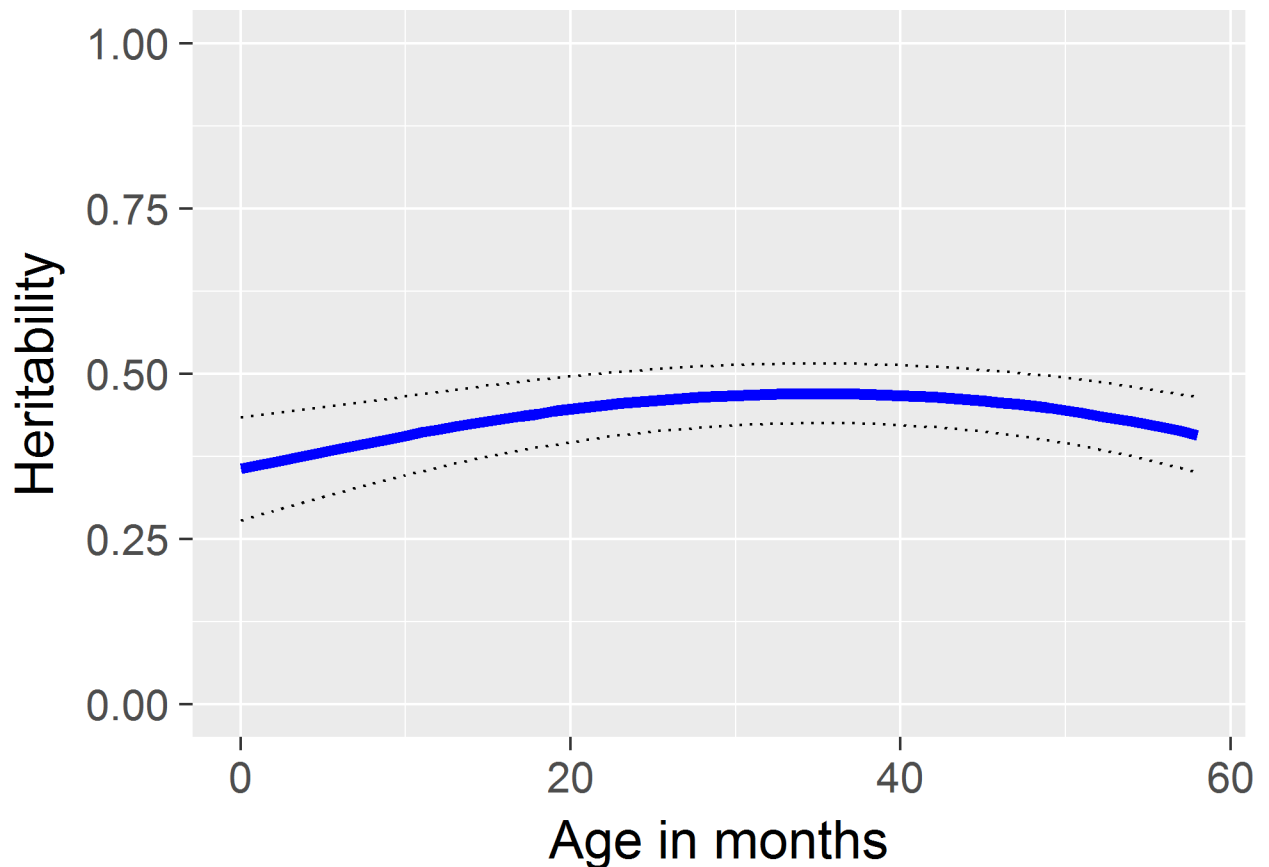


Fig 3. Heritability estimate of RLTL by month of age; standard errors in dotted lines (SE = 0.045–0.078).

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polynomial was fitted to the fixed and the random effect which facilitates interpretation of the results.

The fixed curve as described by the Legendre polynomial captured the expected initial decline of RLTL in early life and a relative stability of RLTL later in life (Fig 1). The curve also illustrates a slight increase of RLTL in later life.

Examples of individual animal RLTL curves are shown in Fig 2. These curves illustrate the change in RLTL with age. The intercept, amount and direction of individual RLTL profiles varied considerably and significantly among the animals in the study (Fig 2). The calculation of eigenvalues revealed that the majority of the difference between individual animal profiles is explained by differences in the intercept (94.7%) while 5.3% are due to different shapes of the curves. Eigenfunctions are shown in S1 File.

Monthly heritability estimates for RLTL ranged from 0.356 to 0.470 (SE = 0.045–0.104) and were slightly higher between 20 and 50 months of age than in the beginning of life or at older ages. Considering the SE, heritability estimates remained relatively stable over life (Fig 3).

The genetic correlation between RLTL measurements at birth and at different stages of the animals' lives are shown in Fig 4. As expected, correlations were very high between RLTL at birth and neighbouring ages but decreased as the interval between the two measurements increased. The minimum correlation was 0.693.

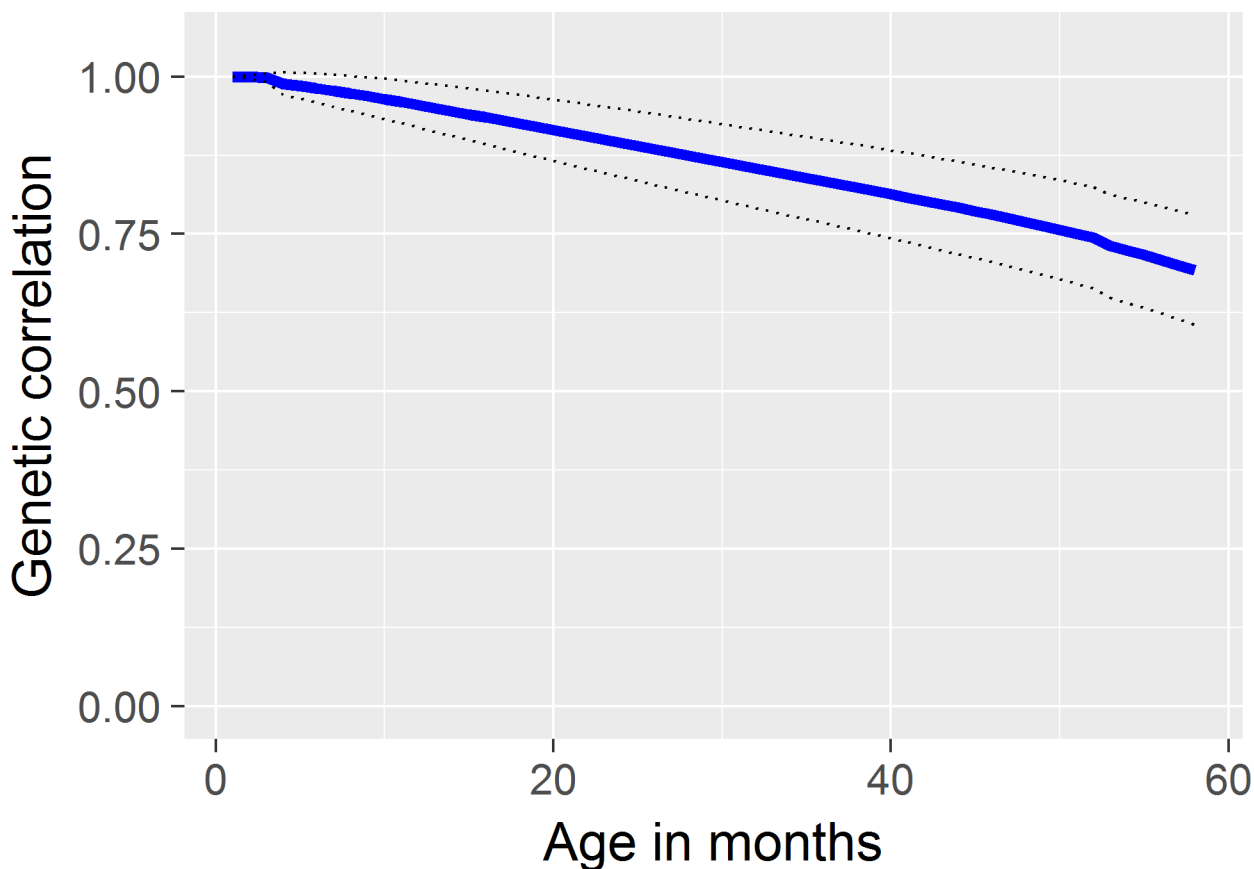


Fig 4. Genetic correlation of RLTL measurements at birth with measurements in later life. standard errors in dotted lines (maximal SE = 0.087).

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Analysis of the association of RLTL dynamics with productive lifespan

Productive lifespan ranged from 17 to 2,823 days (mean = 1,477 days, sd = 76.97 days). To test the association between RLTL profiles (intercept and shape) and productive lifespan, RLTL profiles were clustered into groups depending on the similarity of their RLTL change pattern. Five clusters were formed to capture no telomere change and mild and moderate changes in both directions early in life (attrition vs. elongation). Animals differed more in their intercept than in their direction and amount of change. Of all animals 32% shortened their RLTL slightly in early life, while 29% did not show obvious RLTL change at all (red curve and green curves respectively in Fig 5). Mild elongation early in life was observed in 22% (blue curve in Fig 5). More obvious attrition and elongation early in life was observed in 12% and 5% of the animals, respectively (cyan and pink curves in Fig 5).

The Cox proportional hazard analysis revealed that there was no significant relationship between RLTL profile cluster and productive lifespan ($p = 0.97$) which is visualised in Fig 6.

Discussion

This is the first study exploring individual RLTL profiles of farm animals across time and the largest longitudinal telomere study outside the human literature so far. Our results suggest that individual cattle differ in their RLTL dynamics over life. Although most of the difference between animals is explained by a different average RLTL (intercept) (94.7%), a small

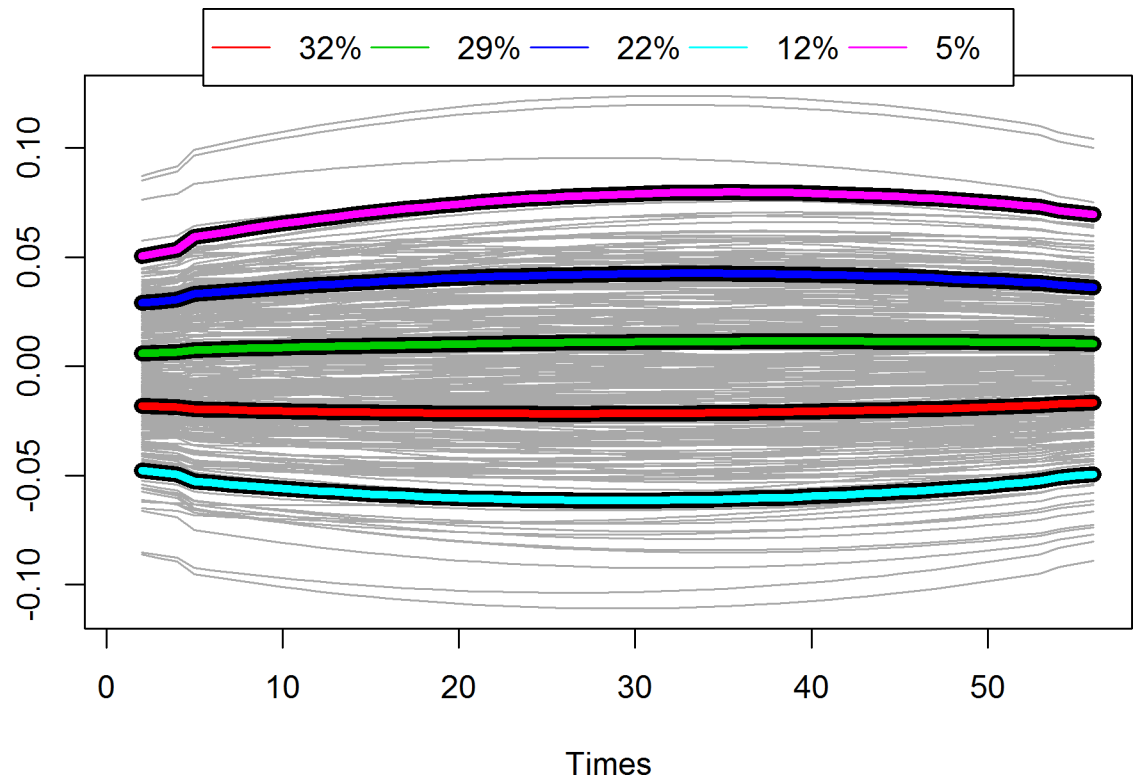


Fig 5. Individual RLTL profiles (grey) and five cluster curves. Of all animals 31% shortened their RLTL slightly in early life (red curve), 30% maintained their RLTL over life (green curve), 22% showed mild elongation in early life (blue curve), 12% more obvious elongation (pink curve) and 4% more obvious telomere attrition (cyan curve).

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proportion is due to different shapes of RLTL profiles (5.3%). This is an important observation that justifies the further investigation of differences in telomere profiles in association with traits of interest such as health, fertility and mortality. The only other study we are aware of that used random regression models for the analysis of longitudinal telomere data did not report a significant difference in telomere dynamics among Seychelles warblers [18], which might have been due to the relatively small sample size of that study.

At a population level RLTL shortened in the beginning of life. The fixed curve calculated in the present study suggests an average RLTL increase later in life. However, this is probably due to the symmetry of a quadratic function and might not reflect biological changes. Therefore, we argue that at a population level telomeres shorten in the beginning of life and remain relatively stable thereafter. Some previous longitudinal studies in baboons and birds support these results, though they did not use random regression models for their analyses [60,61][60,61]. A study in humans found that the early life telomere attrition was followed by a plateau with no telomere change and by a second decline in telomere length as adults grew older [62][62]. It is possible that our study did not include animals that were old enough to show that second decline.

In the present study we report the first heritability estimates for telomere length across all species that were calculated using random regression models. Random regression model estimates do not only inform about the proportion of the variance that is due to additive genetic effects, they also demonstrate how this proportion might change over time. It is known that telomere length is affected by many different genes [22–26]. Epigenetic changes to the genome can alter the translation of genes with ageing [63,64]. If regulatory genes for RLTL were

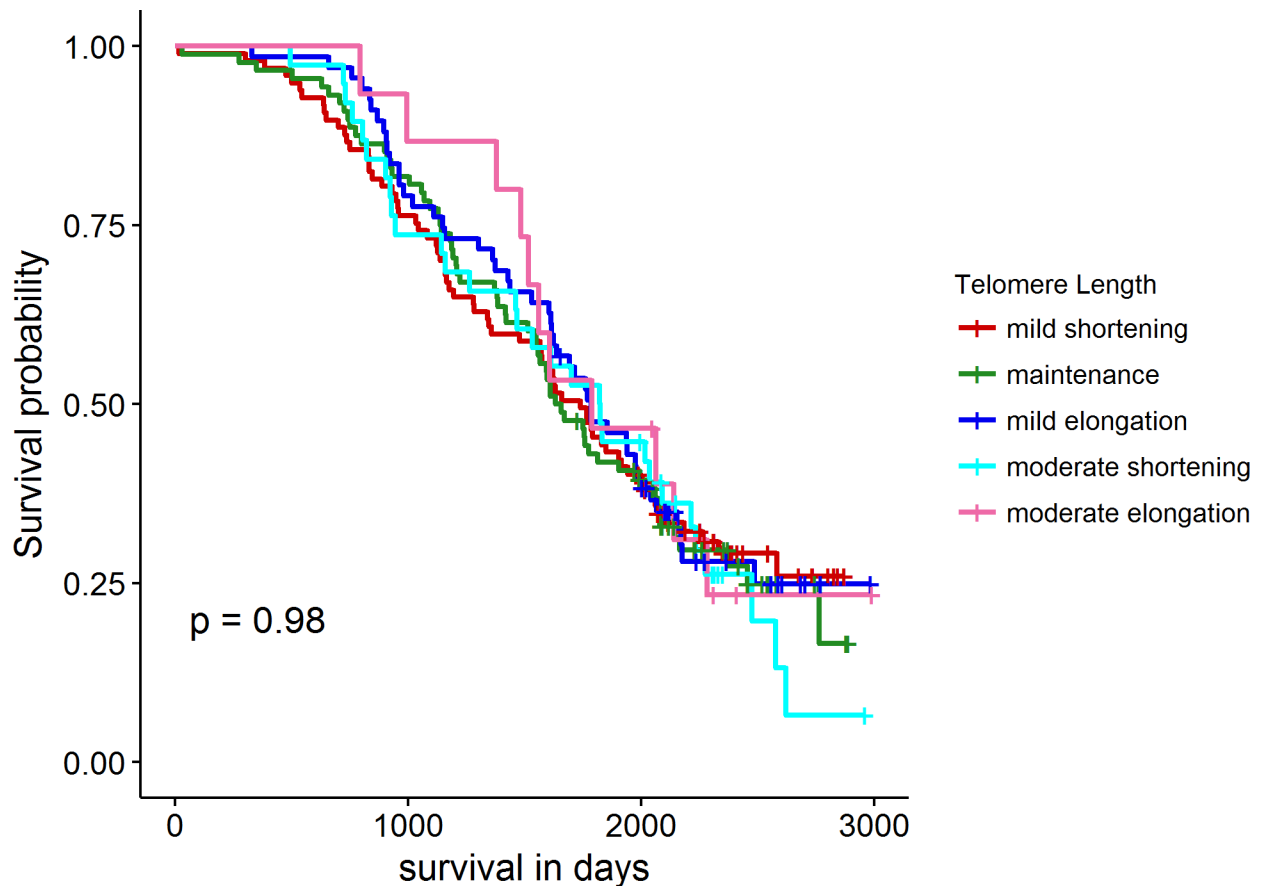


Fig 6. Survival probability of different RLTL profile cluster groups. Colours correspond to colours in Fig 5.

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activated or silenced in an unbalanced manner with ageing, heritability estimates for RLTL might change considerably. However, in the present study we show that heritability estimates for bovine RLTL are not only relatively high (0.36 to 0.47; SE = 0.05–0.10) they are also relatively stable (Fig 3). This means that RLTL at all ages could be influenced by breeding programmes. Heritability of telomere length estimated with relatively simpler models has been reported before in humans (0.39–0.82) [27–31], sand lizards (0.52) [32] and kakapos (0.42–0.77) [33].

Within an animal, the genetic correlation between consecutive RLTL measurements decreased as the time interval between measurements increased. This suggests that RLTL might be under different genetic control at different life stages. As mentioned before, epigenetic changes during ageing [63,64] might inhibit or promote genes that play a role in telomere maintenance. Also, telomeres have been reported to have regulatory functions themselves that act on genes in their close proximity and even in further distance [65–67]. For example, long telomeres form bulky structures that can inhibit transcription of genes in their neighbourhood. When telomeres shorten they unfold and enable the expression of those genes. This is known as telomere positioning effect [65]. Also, shelterin can act as transcription factors and thus regulate gene expression [68].

Not much is known about telomere length and its association with productive lifespan in cattle so far. In cross-sectional studies bovine telomere length declines with age and during the lactation period [35,69,70]. A single study found that animals with shorter telomeres were more likely to be culled within the next year [35]. In the present study we did not find a significant relationship between telomere dynamics and productive lifespan in cattle. Dairy cattle

rarely live until their physiological end of life but are usually culled for fertility, productivity or health reasons. In the introduction we argued that productive lifespan was still biologically meaningful, because animals are not randomly selected for culling. However, the relationship between productive lifespan and RLTL might be different than these relationships in humans or natural animal populations. Also, a relationship between RLTL and productive lifespan in dairy cattle might be there if RLTL change was examined in a different way. RLTL dynamics might be too pulsatile to be exactly described by random regression models. Future studies are required to investigate the best way to analyse longitudinal datasets that include more than two RLTL measurements per animal. While current results did not show a significant correlation between RLTL and productive life at phenotypic level, a further study examining genetic correlation between the two traits is of high interest as it may provide a different result.

Supporting information

S1 File. Complementary information.
(DOCX)

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Writing – review & editing: Luise A. Seeker, Joanna J. Ilska, Androniki Psifidi, Sarah L. Underwood, Jennifer Fairlie, Hannah Froy, Bruce Whitelaw, Mike Coffey, Daniel H. Nussey, Georgios Banos.

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