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The Effect of Telomere Length Variation on Lifetime Productivity Traits in Sheep

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Abstract

Telomere DNA length exhibits an age-related decline in humans and it is emerging as a biomarker for longevity and fitness. Telomere DNA is important for maintaining the proliferative capacity of stem cells as excessive shortening of these structures, which form protective caps on the ends of chromosomes, reduces the body's ability to rejuvenate aging tissue. As telomere DNA length is highly heritable with a significant genetic influence from the paternal genome, we assessed whether variation in telomere DNA length in sheep aged 1 -7 years was correlated with Australian Sheep Breeding Values (ASBVs) for a range of genetic traits. Only weak genetic relationships were detected between telomere length and ASBVs, with the highest genetic associations observed for birth weight, fatness (CFAT) and two wool quality traits (staple strength and coefficient of variation in fibre diameter).

Executive summary

Telomeres are repetitive stretches of DNA which form protective caps on the ends of chromosomes. Every time a cell divides the machinery responsible for replicating the DNA is unable to copy the terminal ends of these caps, so the chromosomes become slightly shorter. In humans, the length of telomeres can shorten by half over a life time. If telomere length drops below a critical threshold then the proliferative capacity of cells rapidly decline and they cease to function. Evidence suggests that this age-related decline in telomere length is a potential biomarker for longevity and fitness. Telomere length in humans has a heritability estimate of 35 - 80%, with a body of evidence indicating that telomere length is paternally inherited. As telomere length is known to shorten in ovine fibroblasts when they are cultured *in vitro*, this project assessed whether telomere shortening in blood leucocytes was evident in sheep aged 1 – 7 years. The primary objectives were to develop a quantitative PCR methodology for accurately measuring telomere length variation in sheep and to determine whether telomere length variation correlated with Australian Sheep Breeding Values (ASBVs), specifically those associated with traits measuring life time productivity.

The Polymerase Chain Reaction (PCR) is a widely used laboratory technique often employed to amplify specific segments of DNA from the genetic material of livestock. Different approaches to collect, store and extract genomic DNA must be taken depending on the intended PCR analysis method being applied. When single nucleotide polymorphisms or microsatellites are detected using qualitative PCR methods (i.e. genotyping assays), genomic DNA samples must be purified to the point where the majority of PCR inhibitors are removed and DNA integrity is sufficient such that copies of the target DNA sequence can be amplified. However, when quantitative PCR techniques are employed to measure copy number variations, as was the case in this project, the DNA must be free from PCR inhibitors and the DNA integrity needs to be high as well as consistent between all the samples. Therefore, before embarking on this project we assessed three approaches for extracting genomic DNA from whole blood or blood spotted onto FTA cards, to determine the most suitable method for measuring copy number variations with quantitative PCR,. All three kits produced genomic DNA with various levels of PCR inhibitors so the DNA had to be diluted in order to accurately measure telomere length variation in sheep. The Ultraclean DNA Blood Isolation Kit (MoBio, USA) was clearly the best method for this project as it yielded high amounts of genomic DNA which were relatively pure compared with the other two kits tested. Unfortunately, there was no correlation between the telomere data obtained from whole blood and the FTA blood cards. This is likely to be due to variable extraction efficiency of DNA from the FTA blood cards, which may be specific to the highly repetitive telomeric DNA but could equally have affected total genomic DNA recovery. We doubt whether copy number variations (CNV) can be accurately measured from blood spotted on FTA cards, and therefore recommend that whole blood should be used for any CNV-type measurements in livestock.

Telomere length variation was assessed in 120 sheep from the Oaklea Genetics stud in South Australia. Telomere length varied by up to 2-fold in these animals which is a considerable difference given that the estimated average length of ovine telomeres is 20kb. However, no evidence of an age-related decline in telomere length was observed in sheep aged 1 – 7. The correlations between ASBVs and telomere length were generally low, with the highest correlations only ranging between 0.1 and 0.2. These ASBVs included: BWT (0.14), CFAT (-0.14), YFDCV (0.14), HDCFV (0.15), AFDCV (0.15), YSS (-0.1), HSS (-0.1) and ASS (-0.1). The original hypothesis of this project centred on the use of a telomere length measurement at 12 months of age to predict life time performance, but based on the current findings this type of test would not be informative. The observation of small correlations with several lifetime productivity traits important to wool suggests that a follow-up study on telomere length variation in Merinos with phenotypic differences in staple strength and coefficient of variation in fibre diameter may be

warranted. A longitudinal study that covered the first 12 months after birth and measured telomere length attrition in blood and skin samples would be the best way to gain further insight into the predictive capability of telomere length variation on lifetime productivity traits in sheep.

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1 Background

1.1 Telomere structure and function

Mammalian chromosomes shorten by a small amount after every cell division. This shortening is associated with a loss of telomere DNA from the terminal ends of chromosomes. Telomeres are highly conserved between eukaryotic species and consist of specialised DNA structures composed of many thousands of copies of the same tandem repeat sequence (TTAGGG). The telomeres protect the ends of chromosomes from irreversible DNA damage as cells throughout the body divide and replicate.

DNA polymerase is unable to copy the ends of chromosomes when a cell divides. Therefore, the activity of the enzyme, telomerase, is essential in reducing the loss of telomere DNA repeats from the ends of chromosomes. In the early stages of life, telomerase is able to maintain the length of the telomeres, but over time the efficiency of this process declines and the telomeres begin to shorten. The progressive loss of telomere DNA occurs to the point where shortened telomere lengths negatively affect the function and viability of cells. This is best illustrated by the observation of an age-related decline in telomere length in cultured cells, termed “replicative senescence”, which precedes a loss of cell function and eventually leads to cell death. In humans, telomere attrition is believed to be a factor in age-related disease pathologies and a key determinant of overall lifespan.

1.2 Measuring telomere length by quantitative Polymerase Chain Reaction (qPCR)

Terminal restriction fragment analysis (TRF) and telomere quantitative fluorescence *in situ* hybridisation (TQ-FISH) were the first techniques used to measure telomere length. The highly repetitive nature of telomeric DNA initially prevented the use of PCR for this purpose. However, Cawthon (2002) overcame the challenge presented by the formation of dimers in the primers designed to hybridize to the telomere repeats by developing a set of telomere-specific primers where every sixth base was mismatched and the 3’ ends of the two telomere-specific primers were unique. As the results obtained with this qPCR technique are highly correlated with the original methods, the use of qPCR to measure telomere length has steadily increased. A major advantage of the qPCR method is that it’s quicker to perform hence more samples can be screened at one time. Several minor changes have recently been made to the original telomere-specific primers (Cawthon, 2009), so that the primers amplify a defined sized amplicon rather than a range of different sized amplicons. However, the overall strategy for generating and analysing telomere qPCR data has not changed. The relative quantification process involves the determination of the amount of telomere DNA (T) and the amount of a single copy gene (S) in a sample. These amounts measure the telomere repeat copy number and single-copy gene copy number. In order to adjust all the samples back to the same quantity of genomic DNA, T is normalised to S by determining the (T/S) ratio for each sample. The factor by which the T/S ratio of the samples differs from a reference DNA sample determines the relative telomere lengths within an experiment (Cawthon, 2002).

A modification to the relative quantification approach for measuring telomere length has been described by O’Callaghan (2008). This method involves the use of DNA fragments for the telomere repeat and the single copy gene in the form of synthetic oligonucleotides. These DNA fragments with known concentrations are used to construct standard curves, so that absolute quantification of telomere length can be performed. The units used in the absolute quantification method are kilobases of telomere DNA per reaction.

1.3 Telomere length variation: evidence for genetic and age differences

There are two main determinants of telomere length (TL): inheritance and age. The inheritance of telomere length was first illustrated when two common laboratory strains with marked differences in their telomere lengths were crossed and F1 progeny displayed a telomere length that was intermediate of their parents (Manning et al., 2002). Similar findings have also been demonstrated in crosses between Mus musculus (~50kb TL) and Mus spretus (~10kb TL; Zhu, 1998). Studies in humans then demonstrated that telomere length is paternally inherited (Njajou, 2007) and that paternal age at birth is associated with telomere length in their offspring (De Meyer, 2007). Individuals within the same age class have a broad range of telomere lengths with evidence suggesting this is genetically determined (Kappei, 2008). In humans, telomere length is reported to have heritability estimates of between 35 – 80% (Atzmon, 2010). This indicates that a significant component of the maintenance of telomere length and hence the effect it has on biological processes is accounted for by the function of genes. The human disease, dyskeratosis congenital (DC), highlights the important role played by telomerase genes in maintaining telomere length. Autosomal dominant mutations in the TERT and TERC genes, which encode different subunits of the telomerase protein, are responsible for dyskeratosis congenital (Chiang, 2010; Goldman, 2005). Haploinsufficiency of telomerase reverse transcriptase activity in DC patients leads to a rapid shortening of telomere length and a reduction in the proliferative capacity of cells. Dyskeratosis congenital causes abnormal skin manifestations, bone marrow failure, pulmonary fibrosis and an increased predisposition to cancer (Armanios, 2005). More recently, a human study involving a cohort of centenarians and their offspring showed that centenarians had a better ability at maintaining telomere length than unrelated controls, which may account for the exceptional longevity in these families (Atzmon, 2010). A SNP haplotype in the hTERT gene was identified in this cohort and found to be associated with longevity and telomere length.

The majority of evidence for an age-related decline in telomere length is based on studies in humans. Not all species exhibit such a decline. The telomeres of mice and other short lived rodents do not decrease with age, but unlike humans, the telomerase gene is expressed in the somatic cells of rodents so telomere length is maintained (Seluanov, 2007). The replicative senescence observed in the somatic cells of longer-lived mammals such as humans, primates, ungulates and carnivores is a consequence of telomere shortening due to the absence of telomerase expression (Gorbunova, 2009). The lagomorphs are the exception to the rule as the somatic cells of this class of mammalian species don't exhibit replicative senescence, even though they lack telomerase activity. All lagomorph species have especially long telomeres so this protects their cells from replicative senescence, as extended culture of somatic cells from rabbits has been shown to result in telomere shortening. It has been hypothesised that replicative senescence in the somatic cells of longer-lived organisms is a cellular defence mechanism used to protect cells from uncontrolled proliferation and cancer (Gorbunova, 2009). Therefore, telomere shortening occurs in all species which don't express telomerase in their somatic cells. The significance of the age-related decline in telomere length is therefore related to overall lifespan, length of the telomeres at birth and the rate of telomere attrition throughout life. In humans, the majority of studies have shown clear evidence for an age-related decline in telomere length with age (Oeseburg, 2010). A reduction in telomere length variation in the healthy oldest old has been observed in several studies, and genetic and environmental factors have been implicated (Halaschek-Wiener, 2008). As fitness in humans is related to telomere length this study sought to investigate whether telomere length variation in sheep may be an effective predictor of lifetime productivity traits.

2 Project objectives

1. Establish optimised procedures for quantifying the amount of telomere DNA in sheep blood samples
2. Investigate the age-related decline in telomere length and establish whether telomere length is genetically regulated in a selection of sheep bloodlines
3. Explore the relationship between telomere length variation and lifetime productivity traits in sheep

3 Methodology

3.1 Blood sampling

Sheep blood samples were collected on-farm at Oaklea Genetics, Mount Gambier, SA by Don Peglar and a local veterinarian. A blood sample from each animal was collected into K_3EDTA Vacuette tubes (Greiner, Germany) and spotted on FTA Elute Microcards (Whatman, USA). The samples were transported overnight via air freight to SARDI – Livestock & Farming Systems, Roseworthy, South Australia.

3.2 Genomic DNA extractions

Three genomic DNA extraction methods were employed on 120 sheep blood samples. Genomic DNA was extracted from whole blood using the Ultraclean DNA Blood Isolation Kit (MoBio, USA) and the DNeasy Blood and Tissue Kit (Qiagen, Germany). Genomic DNA isolated with the Qiagen kit underwent an additional ethanol precipitation step to remove PCR inhibitors. A hole punch was used to obtain four 3mm diameter sections from dried blood spots on FTA blood cards. Genomic DNA was recovered from the FTA card punches with the Gensolve Whole Blood DNA Recovery kit (Genvault, USA) and then purified using the modified method of McClure, 2009.

3.3 PCR primer design

Published primer sequences for 3 real-time PCR assays that have been used extensively in humans to measure telomere length were tested in this study (Table 1; Cawthorn, 2002; Cawthorn, 2009; O'Callaghan et al., 2008). Real-time PCR assays were also designed against three ovine genes, RPLP0, RPP30 and GDF8 (myostatin), that are present in single copy in the sheep genome. These assays were designed with PrimerExpress software (Applied Biosystems, USA) and they were used for normalising the telomere assay data.

3.4 PCR primer testing

The telomere DNA assays and single copy gene assays were tested on a subset of the sheep genomic DNA samples prepared with the MoBio and Qiagen kits. The specificity and PCR efficiency of the assays (Table 1) were assessed on a 4-fold dilution series of sheep genomic DNA (10ng, 2.5ng, 0.625ng, 0.1625ng) using a SYTO9-based reagent (200nM dNTPs and 1.33nM SYTO9 dye (Invitrogen, USA), 3.5mM MgCl2, 1 x AmpliTaq Gold buffer and 0.2u AmpliTaq Gold DNA polymerase (Applied Biosystems, USA)) on a RotorGene3000 real-time PCR machine (Corbett Research, Australia).

3.5 Real-time PCR measurements on genomic DNA extracted from whole blood and FTA cards

Quantitative PCR measurements were performed on 120 sheep genomic DNA samples extracted from whole blood (Qiagen kit and MoBio kit) and FTA blood cards (Gensolve kit). The three sets of samples, referred to herewith as Qiagen gDNA, MoBio gDNA and FTA gDNA, were measured in triplicate using 7ul reaction volumes (PowerSYBR, Applied Biosystems) on a 384 well real-time PCR machine (7900; Applied Biosystems, USA). The MoBio gDNA and FTA gDNA were diluted 1:20 in 10mM Tris-HCl (pH 8.0) prior to qPCR, while the Qiagen gDNA was not diluted. Each 384 well plated contained a standard curve consisting of a 4-fold serial dilution of pooled gDNA (1:4, 1:16, 1:64 and 1:256). The standard curves were used to calculate the PCR efficiency of each real-time PCR assay and they also provided the data for relative quantification. The TelGC, RPLPOf4r4 and Mst_Int2 assays *(Table 1)* were used in this experiment. The PCR cycling parameters for the TelGC assay were: 95C/10mins (1 cycle), 95C/15sec and 49C/15sec (2 cycles), 95C/15sec, 60C/20sec and 72C/40sec (35 cycles). The PCR cycling parameters for the RPLP0f4r4 and Mst_Int2 assays were: 95C/10mins (1 cycle), 95C/15sec, 60C/20sec and 72C/40sec (40 cycles). The qPCR data from all the 384 well plates were analysed using a cycle threshold of 0.1. Tab delimitated text files from each real-time PCR run were exported from the SDS 2.3 software (Applied Biosystems, USA) and these were compiled for the statistical analyses conducted with ASREML by Associate Professor Wayne Pitchford (Department of Animal and Veterinary Science, University of Adelaide, Roseworthy Campus, South Australia).

Gene	Assay name	Primer sequences (5' - 3')
Telomere[2]	Tel1	Forward: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT Reverse: TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA
Telomere[3]	TelGC	Forward: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT Reverse: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Telomere[4]	Tel	Forward: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT Reverse: CGGCGGCGGGCGGCGCGGGCTGGGCGGCTTCATCCACGTTCACCTTG
RPLPO	RPLPOfr	Forward: CCCATTCTATCATCAATGGGTACAA Reverse: CAGCAAGTGGGAAGGTGTAATCA
RPLPO	RPLPOf2r2	Forward: ATTGGTTACCCGACTGTTGC Reverse: CTTTTCAGCAAGTGGGAAGG
RPLPO	RPLPOf3r3	Forward: CTTCCCACTTGCTGAAAAGG Reverse: TGAGCACTCATGGATTTTGG
RPLPO	RPLPOf4r4	Forward: AAATCGCATGTGCTGTTGAG Reverse: CTGTGGAAGGGAGGAAACAG
RPP30	RPP30fr	Forward: CGGACATCAGAAGCAGATGA Reverse: TCAGCTCTCACACTTGGCTTT
RPP30	RPP30f2r2	Forward: TTCCACGGTGAAGAAACCTC Reverse: TTTGCAGGCTGGAAGAGAAT
RPP30	RPP30f3r3	Forward: TTTGATGCAGGTCTGCAAAG Reverse: AAGGCCTTTCTGCAGCACTA
GDF8	oMST_In1	Forward: CAAGGTGAAGGATTGGGAGA Reverse: GCAACCAAATGCAATTAGGAA
GDF8	oMST_In2	Forward: TGGAGTTCGTCTTTCCAACC Reverse: GGAAGGCAGAGTGATGAAGG

Table 1: Oligonucleotide DNA sequences for the telomere DNA and single copy reference gene (RPLP0, RPP30 and GDF8) assays. [2,3,4] For information on the telomere DNA assays refer to the references mentioned in section 3.3.

4 Results and discussion

4.1 Comparison of three genomic DNA extraction methods

Genomic DNA (gDNA) was initially extracted from 120 sheep blood samples using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions. However, tests performed on this gDNA revealed that a considerable number of the samples contained significant levels of PCR inhibitors, which were impeding the quantitative PCR measurements. The Qiagen gDNA was precipitated with ethanol to provide a further level of purification. The precipitated gDNA performed well in qPCR, but the additional purification step was both time consuming and a likely source for unwanted technical variation between the samples. To overcome these problems we moved away from a column-based gDNA extraction method. The 120 sheep blood samples were re-extracted with the MoBio kit, which combined a blood lysis step with a direct nucleic acid precipitation step. Higher gDNA yields were obtained with the MoBio kit and negligible PCR inhibition was detected for this gDNA when it was diluted to a similar concentration as the ethanol precipitated Qiagen gDNA.

A modified method for extracting gDNA from FTA blood cards had to be employed as the method recommended by the manufacturer involved the DNeasy Blood and Tissue kit (Qiagen). The modified method that we employed was published by Jerry Taylor's group (University of Missouri-Columbia, USA; see McClure, 2009). This method has been successfully used to prepare bovine genomic DNA from FTA blood cards for use with the Illumina Bovine SNP50 iSelect BeadChip. Unfortunately, this method turned out to be extremely slow and tedious and is not be suitable for extracting gDNA from large numbers of blood samples. As expected, the amount of gDNA extracted from FTA blood card punches was considerably lower than the amount obtained with the other two methods. Even after phenol-chloroform extraction and an ethanol precipitation step, considerable levels of PCR inhibitors remained in the gDNA extracted from the FTA blood cards. As a consequence, the FTA gDNA had to be diluted 20-fold to reduce the level of PCR inhibitors to a point where they did not interfere with the qPCR kinetics. Since the concentration of FTA gDNA was already quite low, the 20-fold dilution shifted the detection threshold of the single copy reference genes beyond PCR cycle 30 which is the point where qPCR accuracy begins to decline quite rapidly.

The genomic DNA extracted with the MoBio kit was ideally suited to the measurement of telomere DNA length in sheep blood samples. This kit produced high concentrations of genomic DNA using a quick and simple methodology. The labour costs associated with the extraction of genomic DNA from blood samples with the MoBio kit were about half that of the other two methods. However, the simplicity of the MoBio methodology was its main advantage over the other two methods as this greatly reduced the likelihood for DNA yield differences between samples due to technical variation. In contrast, the ethanol precipitation and 70% ethanol washing steps that were applied to the Qiagen gDNA samples are likely to have affected the amount of telomere DNA and/or total genomic DNA recovered from the original preparations. Therefore, the relative abundance of telomere fragments to target gDNA sequences (i.e. RPLP0 and Myostatin) was probably altered in the precipitated Qiagen samples. The ethanol precipitation step is likely to have contributed to the telomere length differences detected between the kits (see MoBio versus Qiagen results in section 4.5).

To our knowledge there have been no reported studies where telomere length has been measured on genomic DNA extracted from FTA blood cards. When designing this study we decided to measure telomere DNA length in whole blood and in the same blood samples spotted on FTA cards to check if the same results could be obtained with these methods. The telomere length data we obtained from FTA cards are different to the telomere length data obtained from whole blood. This result probably reflects why whole blood has been used in all published studies

investigating telomere length. From our experience with FTA blood cards, the actual extraction of genomic DNA off these cards doesn't seem to be the problem because we extracted a reasonably consistent amount of relatively intact gDNA from each sample *(Figure 1)*. Although the integrity of the gDNA from FTA cards was good the purity was extremely poor, containing high concentrations of PCR inhibitors. A 20-fold dilution of the FTA-extracted DNA was required to reduce the PCR inhibitors to sub-inhibitory levels, which meant that the gDNA concentration was barely sufficient for the qPCR quantification. So, while it was possible to use the FTA gDNA for telomere length measurements, the marked discrepancies between this data and the telomere length data from whole blood raises questions about the efficiency and variability associated with extracting highly repetitive telomere DNA from these cards.

In summary, our results indicate that sheep telomere length is best measured on whole blood samples rather than blood spotted onto FTA cards. The telomere length qPCR data for all three genomic DNA extraction methods are presented in this report so that comparisons can be made between the data. However, we only consider the MoBio preparations as truly representing the telomere lengths of the 120 sheep examined in this study.

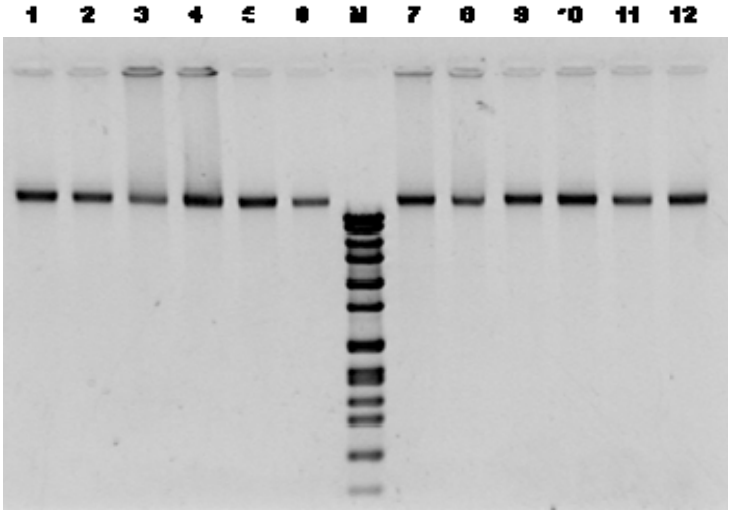

Figure 1: Agarose gel electrophoresis (1%) of 12 sheep genomic DNA samples extracted from dried blood spots on FTA elute microcards. Lane M contains SPPI/EcoRI DNA markers (Geneworks, Adelaide).

4.2 Assessment of telomere and single-copy gene real-time PCR assays

The published primer sequences for three telomere assays were tested on a subset of sheep genomic DNA samples. The TelGC telomere assay (Cawthorn, 2009) was selected over the Tel1 (Cawthorn, 2002) and Tel (O'Callaghan et. al. 2008) assays. The TelGC assay was more sensitive and the nature of the primer design which amplifies a defined sized PCR product *(Figure 2)* is advantageous when calculating the actual PCR amplification efficiency. The best assay for each of the single copy genes was selected based on their qPCR sensitivity and reproducibility. The RPLP0f4r4 and myostatin intron 2 (Mst_Int2) assays were selected as the single-copy genes for the qPCR analysis of telomere length in sheep.

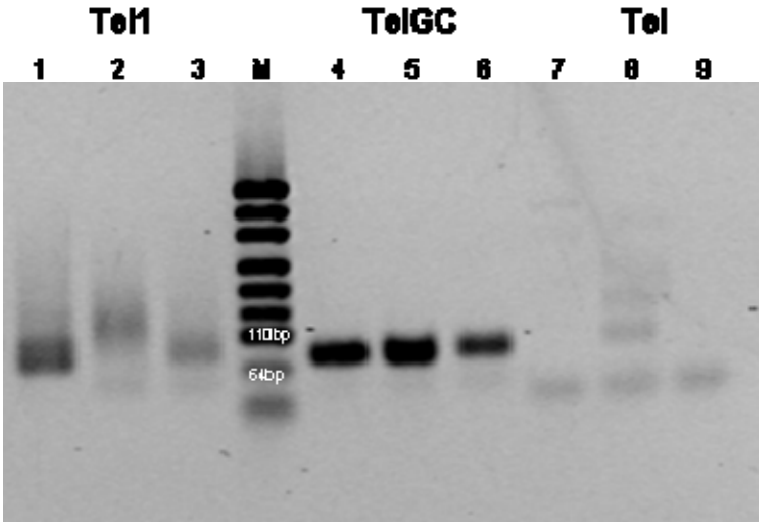

Figure 2: Agarose gel electrophoresis (3%) of the Tel1 (lanes 1-3), TelGC (lanes 4-6) and Tel (lanes 6-9) PCR products amplified from sheep genomic DNA using 2mM MgCl2, 3.5mM MgCl2 and 3.5mM MgCl2 + 1M betaine for each primer set. Lane M contains pUC19/HpaII DNA size markers (Geneworks, Adelaide). The expected size of the TelGC amplicon is 79bp. The size of the DNA markers above and below the TelGC amplicon is shown above.

4.3 General comments regarding the qPCR data used to assess telomere length

The TelGC, RPLP0 and myostatin qPCR data on the MoBio samples were generated on the 30th of October and the 13th of November, 2010. The qPCR data for the Qiagen and FTA samples were generated on the 13th of November, 2010. A total of 6 replicates of qPCR data were generated on the three sets of genomic DNA. As the focus of the telomere analysis was on the MoBio samples, replication of this data was performed both within and across day. The TelGC assay was replicated on the same day for the Qiagen samples, but the FTA samples were only measured once.

Human telomere length studies all use the RPLP0 gene as the single-copy gene. We were quite amazed to find clear evidence for RPLP0 pseudogenes in the genomes of human and bovine whilst designing primers against the ovine version of this gene. To ensure that our RPLP0 assays were unable to amplify the intron-less RPLP0 pseudogene(s) in sheep, the ovine primers were designed to intronic sequences within the RPLP0 gene. In addition, to check that the qPCR data obtained from the ovine RPLP0 gene was consistent with that of a single-copy gene, assays targeting the well–characterised myostatin gene, which is present in the ovine genome in a single copy, were also used in this study. The correlation between the RPLP0 and myostatin genes confirmed that these assays were detecting single copies of each gene *(Figure 3)*. Interestingly, the short comings associated with some of the published telomere length data are the subject of a recent review (Horn, 2010).

The correlation between the RPLP0 and myostatin assays was lower than desired when the genomic DNA samples were measured on a 384 well real-time PCR system *(Figure 3)*. The same correlations were higher during the initial primer testing phase which was performed on a RotorGene3000. Shifting the RPLP0 assay directly from one real-time PCR system to another without first optimising the cycling conditions on the second machine is the likely cause for the increased technical variation observed for the RPLP0 assay. The statistical analysis on the qPCR data, described in section 4.4, confirms our observations of reproducibility problems with the RPLP0 data. Therefore, the telomere length data for the 120 sheep was normalised using

only the myostatin data as a covariate. Although two single copy reference genes are not required for the normalisation of telomere length data, we thought this approach would provide a more accurate normalisation strategy than using a T/S ratio produced from only one single-copy gene. Whilst sensitivity is a key feature of qPCR, a potential downside is that DNA is quantified in the PCR log phase, so modest variations in the cycle threshold readings can have profound effects on the accuracy of the data. Therefore, greater replication of the assays generally improves the overall accuracy of the data.

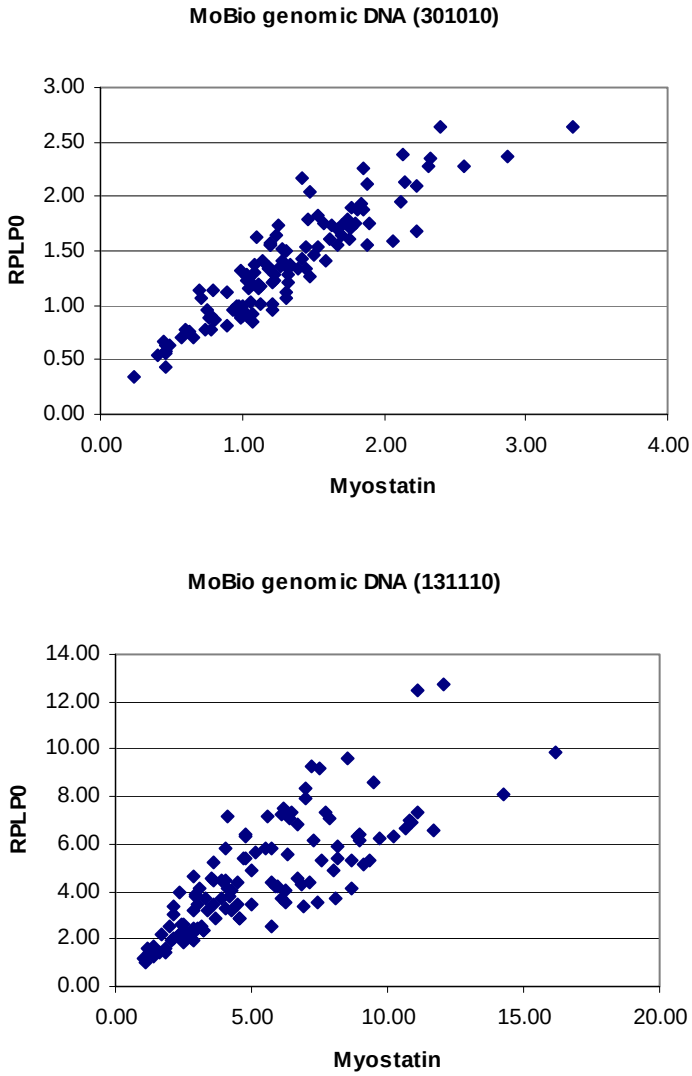

Figure 3: Correlation between the RPLP0 and Myostatin qPCR assays measured on the MoBio DNA samples on 301010 and 131110.

4.4 Genetic relationship between telomere length and lifetime productivity in sheep

Two mixed models were fit to the telomere data. After initial analysis, it became clear that it was necessary to log-transform the telomere data. Since the genomic data (RPLP0 and Myostatin) were also generated from a multiplicative process, they were also transformed. The residuals from the analysis appear normally distributed with no obvious problems *(Figure 4)*.

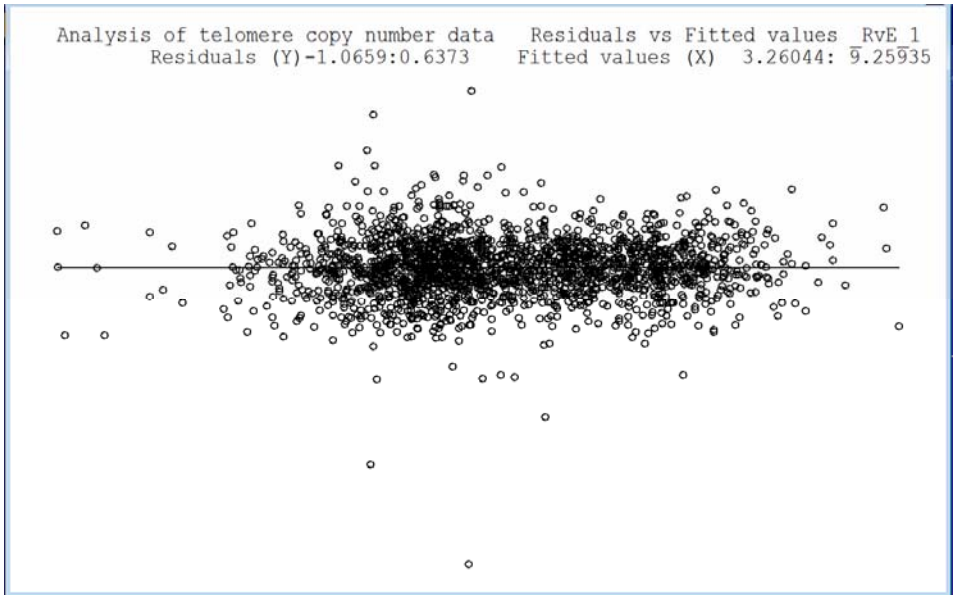

Figure 4: Scatterplot of residuals against fitted values as generated by ASREML.

Model 1 contained fixed effects of the two covariates (RPLP0 and Myostatin), main effect of replicate (6 levels), the interaction between replicate and the covariates (allows for separate slopes), then random effects of rep.id. A variance structure was then placed on the random effect so that 6 separate variances were estimated for each replicate and all 15 covariances were also estimated, resulting in a correlation matrix. Replicate means are presented in *Table 2* and the correlation matrix in *Table 3*.

Model 2 partitioned the fixed replicate effect into its components: sample (whole blood or FTA card), kit (MoBio, Qiagen, FTA), day (30th October or 13th November) and replicate on a given day (2 for MoBio on 30/10 and 2 for Qiagen on 13/11). The random effect was also partitioned to demonstrate the sources of variance in telomere copy number between animals. The analysis of variance is presented in *Table 4* and variance estimates in *Table 5*.

Sample	Kit	Day	Rep	Telomere copy number	Approximate SEM*	Approximate CV*
Blood	MoBio	30/10	1	342	7	2.0
Blood	MoBio	30/10	2	410	9	2.2
Blood	MoBio	13/11	3	685	16	2.3
Blood	Qiagen	13/11	4	1008	37	3.7
Blood	Qiagen	13/11	5	1090	61	5.6
Card	FTA	13/11	6	534	36	6.7

Table 2: Mean copy number for replicates. *Values are back-transformed from natural log so are not normally distributed on this scale. Approximate CVs calculated as SEM/mean.

	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
Rep 1	0.0386	0.88	0.74	0.29	0.39	0.02
Rep 2	0.0274	0.0252	0.70	0.18	0.34	0.12
Rep 3	0.0327	0.0251	0.0509	0.43	0.44	0.06
Rep 4	0.0145	0.0072	0.0247	0.0654	0.63	0.09
Rep 5	0.0316	0.0217	0.0406	0.0657	0.1660	0.13
Rep 6	0.0028	0.0113	0.0088	0.0137	0.0325	0.3612

Table 3: RPLP0 and Myostatin normalised telomere data. Variances* (on diagonal), covariances* (below diagonal) and correlations (above diagonal) between methods *(Co)variances in units of $\log_n$(telomere copy number)^2

Source of variation	Degrees of Freedom	F-value based on Wald test
RPLP0	1	1602.80
Myostatin	1	255.28
Sample	1	3.65
Sample.Kit	1	535.40
Sample.Kit.Day	1	1028.49
Sample.Kit.Day.Rep	2	22.50
Sample.RPLP0	1	28.43
Sample.Myostatin	1	14.89
Sample.Kit.RPLP0	1	7.39
Sample.Kit.Myostatin	1	52.45
Sample.Kit.Day.RPLP0	1	52.68
Sample.Kit.Day.Myostatin	1	9.48
Sample.Kit.Day.Rep.RPLP0	2	2.57
Sample.Kit.Day.Rep.Myostatin	2	2.40

Table 4: Analysis of variance

Source	Model terms	Variance component	Component /SE*	Variance (%)
Animal	135	0.01089	0.9	7
Sample.Animal	270	0.05742	3.2	34
Sample.Kit.Animal	810	0.06443	5.8	39
Sample.Kit.Day.Animal	1620	0.00000	Zero	0
Sample.Kit.Day.Rep.Animal	9720	0.01856	9.7	11
Residual	2058	0.01602	25.2	10

Table 5: Variance components. *Very approximate test of significance of the variance component

Shows that the between animal variation is different for the samples (whole blood versus FTA cards) and for kits (MoBio versus Qiagen). The residual variance represents the within animal variance (between plate) and was only 10% of the total, indicating a low technical variability and therefore high repeatability.

The final analysis was to estimate correlations between ASBVs and the telomere length estimates, using both the RPLP0 and myostatin qPCR data as covariates. This was done using the BLUPs for individual telomere length from Model 1 and is presented in *Table 6*.

Trait	rep 1	rep 2	rep 3	rep 4	rep 5	rep 6
YOB	-0.02	-0.03	-0.01	0.09	0.09	0.10
BWT	0.14	0.13	0.07	-0.09	-0.09	-0.01
MBWT	-0.08	-0.09	-0.08	-0.06	-0.05	-0.18
WWT	0.17	0.13	0.12	-0.05	-0.08	-0.02
MWWT	-0.05	-0.08	-0.06	-0.03	-0.01	-0.14
PWWT	0.13	0.09	0.11	0.01	-0.04	0.01
YWT	0.09	0.03	0.05	0.04	0.00	-0.02
HWT	0.07	0.05	0.03	-0.06	-0.09	0.00
AWT	0.05	0.03	0.00	-0.05	-0.06	-0.02
PFAT	-0.03	-0.01	-0.03	0.19	0.13	-0.05
CFAT	0.01	0.04	0.02	0.18	0.13	-0.08
HFAT	-0.04	-0.02	-0.03	0.18	0.15	-0.05
YFAT	-0.02	0.01	0.00	0.16	0.13	-0.09
PEMD	0.02	0.04	0.04	0.29	0.16	-0.02
YEMD	0.03	0.06	0.05	0.26	0.12	-0.03
HEMD	0.01	0.04	0.04	0.27	0.14	-0.01
NLW	-0.14	-0.10	-0.16	-0.16	-0.01	0.06
NLB	-0.14	-0.11	-0.16	-0.13	-0.02	0.02
YGFW	0.03	0.03	0.00	-0.06	-0.03	0.06
HGFW	0.05	0.05	0.00	-0.07	-0.05	0.03
AGFW	0.04	0.06	0.00	-0.10	-0.05	0.03
YFD	0.02	0.02	0.02	0.00	0.06	0.02
HFD	-0.02	-0.02	-0.02	0.02	0.07	0.03
AFD	-0.01	-0.02	-0.01	0.03	0.08	0.03
WFEC	0.06	0.02	0.07	-0.03	-0.05	-0.13
PFEC	0.05	0.02	0.07	-0.03	-0.06	-0.12
YFEC	0.04	0.01	0.04	-0.06	-0.06	-0.11
YCFW	0.04	0.05	0.01	-0.09	-0.04	0.06
HCFW	0.05	0.06	0.01	-0.08	-0.04	0.04
ACFW	0.03	0.04	0.00	-0.08	-0.03	0.02
YFDCV	0.10	0.12	0.05	-0.18	-0.15	-0.01
HFDCV	0.09	0.11	0.05	-0.17	-0.12	-0.01
AFDCV	0.10	0.11	0.05	-0.15	-0.11	-0.02
YSC	0.00	-0.06	-0.01	0.12	0.03	-0.03
HSC	0.01	-0.05	0.02	0.11	0.02	-0.03
YSL	-0.03	0.01	-0.04	-0.10	0.01	0.03
HSL	-0.02	0.01	-0.06	-0.10	-0.02	0.01
ASL	-0.01	0.03	-0.04	-0.09	0.01	0.00
YSS	-0.13	-0.16	-0.06	0.18	0.17	0.05
HSS	-0.12	-0.15	-0.06	0.18	0.17	0.03
ASS	-0.12	-0.15	-0.05	0.18	0.16	0.04
MGFW	-0.13	-0.15	-0.12	-0.02	-0.04	-0.16
MCFW	-0.13	-0.15	-0.12	-0.02	-0.04	-0.16
COEFF_IB	0.02	0.00	0.10	0.08	0.05	0.05
Border$	-0.06	-0.05	-0.09	-0.04	0.03	0.02
SRC	-0.07	-0.05	-0.10	-0.04	0.03	0.03

Carcase+	0.13	0.10	0.12	0.05	-0.03	0.02
Lamb2020	0.10	0.10	0.08	0.19	0.08	0.05
Coopworth	-0.06	-0.05	-0.09	-0.04	0.03	0.02

Table 6: Correlations with ASBVs using RPLP0 and myostatin as covariates

A subsequent analysis was conducted using model 1 but with only myostatin included as a covariate. The correlation between telomere length replicates on the same day from this model is very high (0.97, *Table 7*) and between days for MoBio is much higher than previous (0.82 versus 0.74 or 0.70, *Table 7* versus *Table 3*).

	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
Rep 1	0.1314	0.97	0.82	0.26	0.30	0.01
Rep 2	0.1041	0.0885	0.82	0.21	0.28	0.05
Rep 3	0.0997	0.0815	0.1122	0.31	0.37	0.05
Rep 4	0.0310	0.0211	0.0349	0.1109	0.81	0.17
Rep 5	0.0512	0.0387	0.0582	0.1271	0.2202	0.22
Rep 6	0.0016	0.0122	0.0133	0.0438	0.0795	0.5952

Table 7: Myostatin normalised telomere data. Variances* (on diagonal), covariances* (below diagonal) and correlations (above diagonal) between methods *(Co)variances in units of $\log_n$(telomere copy number)^2

The correlations were re-calculated between ASBVs and the telomere length estimates, using only the myostatin qPCR data as the covariate. This was done using the BLUPs for individual telomere length from Model 1 and is presented in *Table 8*. The greatest correlations with the MoBio kit are birth weight (BWT), fatness at the C site (CFAT), coefficient of variation in fibre diameter (YFDCV, HFDCV, AFDCV) and staple strength (YSS, HSS and ASS). As these correlations are all low they could just be artefacts.

Table 8: Correlations with ASBVs using myostatin as a covariate.

Trait	rep 1	rep 2	rep 3	rep 4	rep 5	rep 6
YOB	0.05	0.04	0.03	0.13	0.13	0.12
BWT	0.10	0.11	0.17	0.04	0.13	0.05
MBWT	-0.01	0.00	0.01	-0.12	-0.04	-0.06
WWT	-0.05	-0.06	-0.04	-0.07	-0.02	0.05
MWWT	-0.05	-0.06	-0.06	-0.07	-0.01	-0.09
PWWT	-0.05	-0.07	-0.05	-0.01	-0.01	0.07
YWT	-0.03	-0.05	-0.07	0.01	-0.03	0.07
HWT	-0.07	-0.07	-0.05	-0.08	-0.08	0.07
AWT	-0.06	-0.06	-0.07	-0.04	-0.04	0.09
PFAT	-0.04	-0.03	-0.09	-0.05	-0.10	-0.05
CFAT	-0.12	-0.12	-0.15	-0.10	-0.12	-0.06
HFAT	-0.04	-0.05	-0.11	-0.04	-0.10	-0.06
YFAT	-0.04	-0.04	-0.09	-0.03	-0.08	-0.07
PEMD	-0.03	-0.02	-0.05	0.00	0.00	-0.01
YEMD	-0.05	-0.03	-0.04	-0.01	0.00	0.00
HEMD	-0.06	-0.04	-0.05	0.00	0.00	0.00
NLW	0.03	0.04	0.09	0.08	0.08	0.04
NLB	0.01	0.02	0.06	0.09	0.08	0.02
YGFW	0.10	0.08	-0.01	0.05	-0.06	0.00
HGFW	0.09	0.08	0.00	0.01	-0.08	0.01
AGFW	0.10	0.10	0.02	0.01	-0.08	0.02
YFD	0.00	-0.01	-0.07	0.07	-0.01	0.02
HFD	0.01	0.00	-0.07	0.08	0.00	0.03
AFD	0.00	-0.01	-0.08	0.09	0.01	0.04
WFEC	-0.01	-0.01	-0.04	-0.07	-0.08	-0.08
PFEC	0.01	0.00	-0.04	-0.03	-0.07	-0.08
YFEC	0.01	0.00	-0.05	-0.04	-0.09	-0.09
YCFW	0.09	0.08	0.00	0.03	-0.07	-0.01
HCFW	0.08	0.08	0.00	0.01	-0.08	0.00
ACFW	0.11	0.10	0.03	0.02	-0.06	0.01
YFDCV	0.15	0.14	0.13	-0.07	-0.10	-0.09
HFDCV	0.17	0.16	0.14	-0.03	-0.08	-0.09
AFDCV	0.17	0.16	0.14	-0.04	-0.10	-0.10
YSC	0.00	-0.03	-0.02	0.07	-0.04	0.06
HSC	-0.01	-0.04	-0.03	0.08	0.00	0.09
YSL	0.05	0.06	0.00	0.06	-0.02	0.02
HSL	0.05	0.06	0.00	0.01	-0.06	0.02
ASL	0.06	0.07	0.00	0.03	-0.03	0.04
YSS	-0.09	-0.10	-0.12	0.18	0.15	0.13
HSS	-0.10	-0.11	-0.12	0.17	0.14	0.12
ASS	-0.10	-0.11	-0.13	0.18	0.14	0.12
MGFW	-0.04	-0.05	-0.06	-0.11	-0.07	-0.08
MCFW	-0.04	-0.05	-0.06	-0.11	-0.07	-0.08
COEFF_IB	0.16	0.15	0.11	0.12	0.09	0.13
Border$	-0.04	-0.03	0.02	0.07	0.10	0.06
SRC	-0.05	-0.04	0.02	0.05	0.09	0.07
Carcase+	-0.04	-0.04	-0.01	0.03	0.06	0.08
Lamb2020	-0.06	-0.06	-0.04	0.01	0.04	0.09
Coopworth	-0.04	-0.03	0.02	0.07	0.10	0.06

4.5 Telomere length in sheep did not exhibit an age-related decline

There was no evidence of a decline in telomere length with increasing age within any of the three batches of sheep genomic DNA (MoBio, Qiagen and FTA) examined in this study. Irrespective of animal age, the variation in telomere length between animals within a particular age group appeared similar *(Figure 5)*. With specific reference to the MoBio data, telomere length within each age group varied about the mean by 30-50%. Telomere length variation in the MoBio data is generally comparable with the variation reported in human telomere lengths within age groups *(Figure 6)*. Telomere length variation detected in sheep aged between 1-7 years in this study was similar to the variation reported for sheep aged 1-3 years in the only other comparable study conducted on sheep (Alexander, 2007). The primarily focus of the Alexander (2007) study was telomere shortening in cultured fibroblasts, somatic cell derived sheep clones and their offspring. Included in the Alexander (2007) paper was an assessment of *in vivo* telomere length shortening in skin biopsies from Dorset cross sheep aged 1- to 36-months old. The terminal restriction fragment method (TRF) was used and telomere length was shown to decline with age (*Figure 7*; $r = -0.442$, $p < 0.05$). Based on the linear regression equation, the authors estimated that telomere length in sheep declined by approximately 1kb per year.

A closer inspection of the telomere length data in the Alexander (2007) paper shows that the greatest decline in telomere length occurred between the 6 month and 12 month measurements. However, telomere length between 1-3 years of age appeared to remain unchanged, and the variation between these animals was similar to the findings in the current study. Therefore, it would appear that telomere length in the skin of sheep declines in the first year of life and remains constant for the next couple of years, and similarly the telomere length in blood leukocytes remains stable in sheep between 1–7 years. Although we are unable to show whether telomere length in sheep blood leukocytes decreases in the first 12 months of life as appears evident from the sheep skin data, two longitudinal studies conducted in baboons (Baerlocher, 2007) and cats (Brummendorf, 2002) would suggest that this is probably the case. In baboons, telomere length in the hematopoietic stem cells of 4 animals declined 2-3kb in the first year of life with negligible attrition observed over the next three years. Even though two baboons had an average telomere length of ~25kb at birth and the other two were only ~15kb, the telomeres of all fours animals declined by a similar amount. The longitudinal data from two kittens showed an even steeper decline in telomere length (~15kb) in the first year of life. Both kittens had a similar telomere length at birth (70kb), which is 5-10 fold longer than humans, but no data was collected beyond the first 12 months of life so the extent that telomere length stabilises in cats after this period remains unknown. However, taken together, these longitudinal studies lend support to the notion that telomere length declines quite significantly early in life before stabilising and declining at a far less significant rate over the next few years. Whether the rate of telomere attrition then increases at some point in the aging process of mammals is not clear, although the data compiled on humans appears to show this pattern of telomere attrition. .

Tissue type differences could provide a plausible explanation for the discrepancies between telomere attrition with age in sheep skin and blood leukocytes. A study examining loggerhead turtles, a long lived mammal, found no evidence for telomere shortening with age in blood leucocytes but did find evidence of telomere shortening in the epidermis (Hatase, 2008). Therefore, while blood and skin cells are both actively dividing throughout life, the telomeres of skin cells may decline more rapidly due to higher rates of cell turnover or alternatively the loss of telomere length with each cell division may be greater in skin than hematopoietic cells. If this were to be true in the skin of sheep, telomere length measured in blood leukocytes may not correlate as well with the life time productivity for wool growth traits such as staple strength or coefficient of variation as direct measurements conducted on skin.

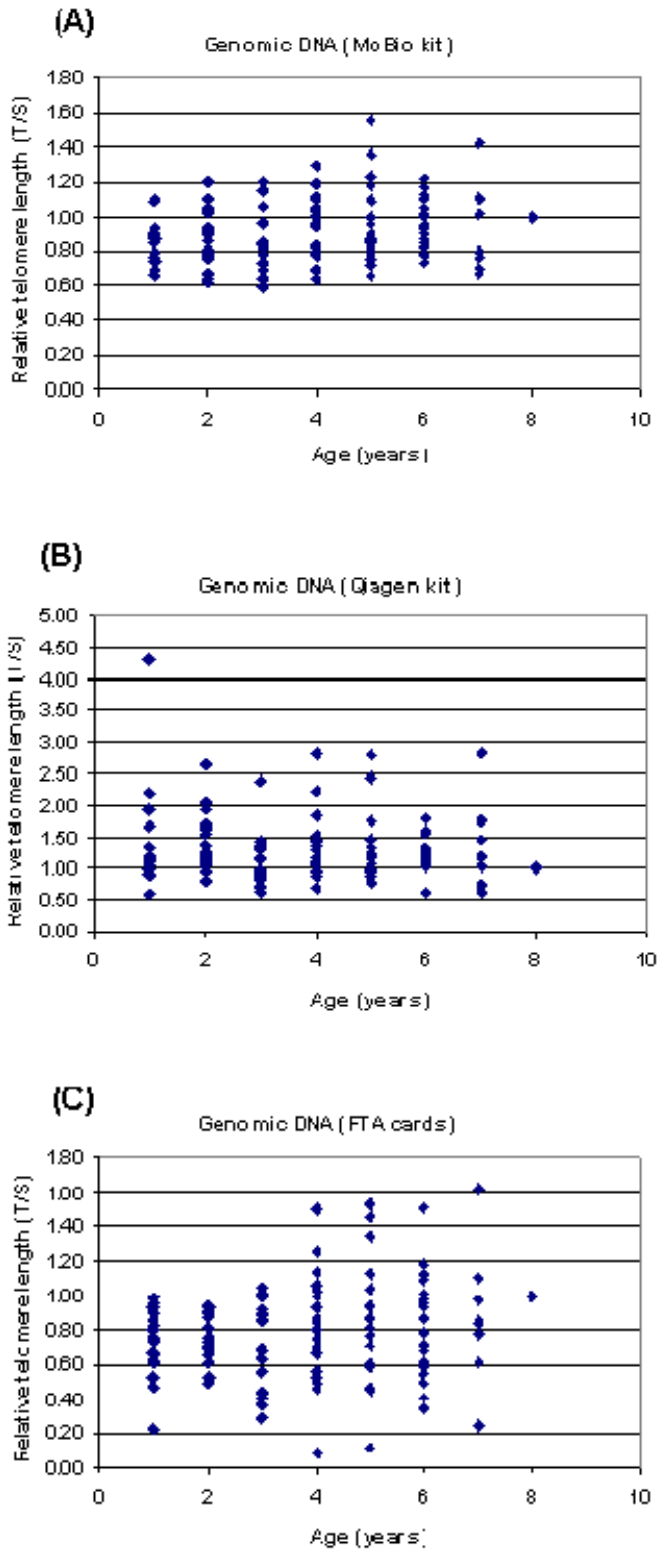

Figure 5: Relative telomere length plotted against sheep age for (A) MoBio, (B) Qiagen and (C) FTA genomic DNA samples. The TS ratio of a reference sample, an 8 year old sheep, was set to 1.00 and all the samples within each DNA extraction set were expressed relative to this value.

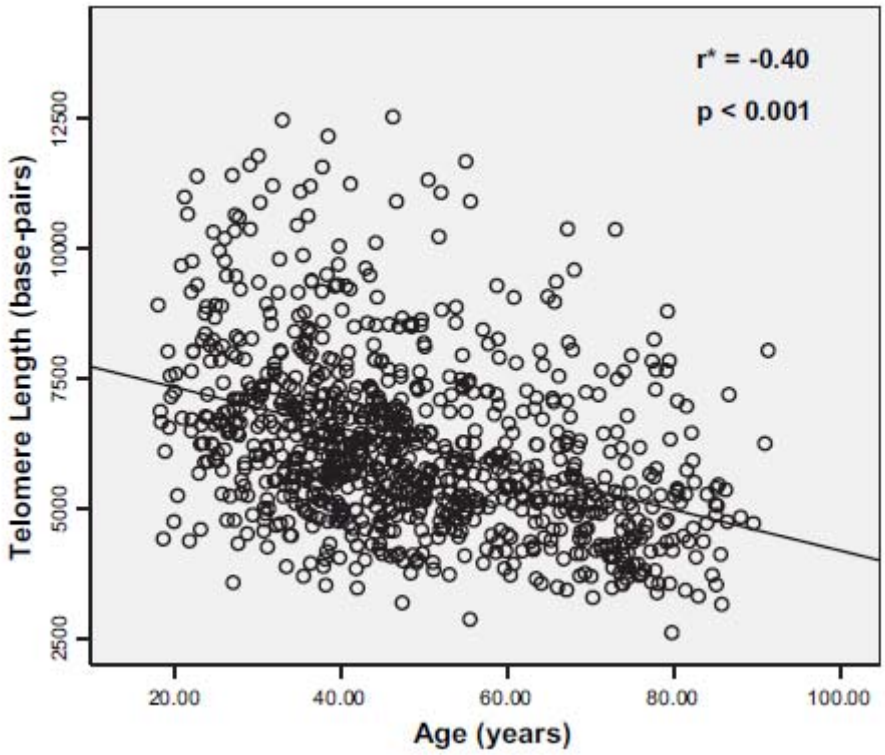

Figure 6: Human telomere length plotted against age. Data was generated on Amish families from Pennsylvania (USA) using 356 men and 551 women. Within this data there were 62 Father-son, 102 Father-daughter, 63 Mother-son and 105 Mother-daughter combinations. *Image reproduced from Njajou et al. 2007.*

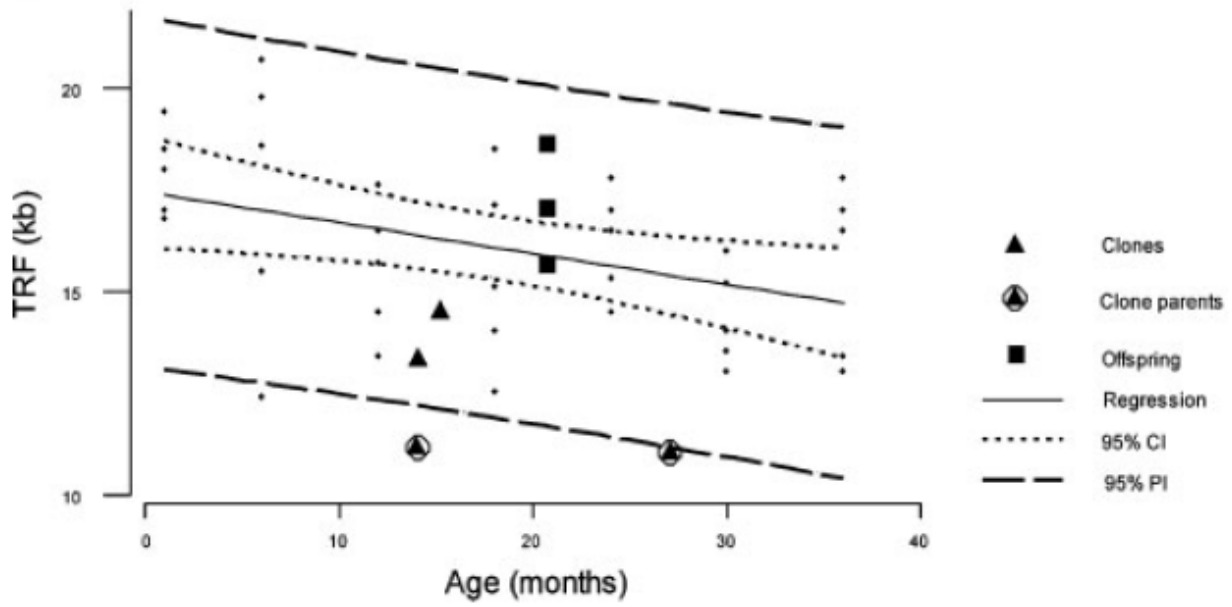

Figure 7: Regression analysis of telomere length in 35 Dorset cross sheep, aged 1, 6, 12, 18, 24, 30 and 36 months. The best-fit regression line, confidence intervals (CI) and predictable intervals (PI) are shown. *Image reproduced from Alexander et al. (2007).*

5 Success in achieving objectives

All the objectives in this project have been met.

1. Establish optimised procedures for quantifying the amount of telomere DNA in sheep blood samples

We have shown that telomere length in sheep is best measured using whole blood, and that the MoBio kit produces high yields of genomic DNA which are ideally suited to the measurement of DNA copy number variations by qPCR. The TelGC assay was identified as the best of three published telomere assays for measuring telomere length in sheep. Our strategies of targeting the intronic regions of single-copy genes for assay design and using multiple covariates to normalise telomere length data are major improvements over the methodology used in humans.

2. Investigate the age-related decline in telomere length and establish whether telomere length is genetically regulated in a selection of sheep bloodlines

   In sheep aged 1–7 years we were unable to detect an age-related decline in telomere length. Given the low variability of telomere length and the small sample size in this experiment we were unable to establish whether telomere length is genetically regulated

3. Explore the relationship between telomere length variation and lifetime productivity traits in sheep

The correlations between the telomere length in blood leukocytes of sheep and Australian Sheep Breeding Values (ASBVs) were low. Telomere length variation measured at 12 months of age is unlikely to be a useful early indicator of lifetime productivity in sheep

6 Impact on meat and livestock industry – Now and in five years time

The molecular diagnostic tools developed in this project will have limited impact in helping to improve lifetime productivity in sheep because the genetic correlation between sheep telomere length and ASBVs appears to be weak. However, technical aspects related to this project could be extremely useful for the development of future molecular diagnostic tools which explore the relationship between copy number variations (CNV) in the sheep genome and important genetic traits. A core consideration in this project was to ensure that the methodology we developed could be readily scaled to handle potential follow-up studies involving 1000 -10000 animals. In achieving this objective the livestock industry is currently equipped with the tools and expertise required to conduct large-scale studies on the relationship between copy number variations and complex genetic traits. As DNA sequencing technology continues to evolve rapidly, an expanding list of ovine copy number variations will be identified over the coming years. A significant expansion in the scale of CNV studies in livestock could readily be achieved using the microfluidic real-time PCR system in our laboratory (BioMark, Fluidigm), which has the capacity to perform 9216 qPCRs per run. In addition, the same technology can perform absolute quantification of copy number variations using a technique known as digital PCR. So, while this project has not progressed beyond the proof-of-concept stage, we have established a robust quantitative PCR methodology that could be widely applied to investigate the emerging genetic relationships between CNVs and phenotypic variation in complex production traits.

7 Conclusions and recommendations

It was hypothesised that a genetic relationship might exist in sheep between the length of their telomeres and lifetime productivity traits. Unfortunately, the results of this study did not provide the solid evidence we hoped to uncover to demonstrate a link between telomere length and lifetime productivity traits in sheep. Since the key goal of this project was to undertake a preliminary investigation into the feasibility of obtaining a telomere length measurement from a relatively young sheep for the purpose of accurately predicting its lifetime productivity at an early age, we can report that such a diagnostic test does not appear to be possible. However, a better predictor of lifetime productivity might be obtained from the rate of telomere length attrition occurring in the first 12 months of life. Evidence from a limited number of longitudinal studies as well as other observations in humans indicate that a rapid decline in telomere length occurs in the first 12 months after birth. Telomere length then remains relatively stable for a prolonged period before declining more rapidly during the onset of old age. As there is already evidence in the literature for a decline in telomere length in the skin of Dorset cross sheep in the first 12 months of life, and two wool quality traits were among the highest genetic correlations detected in this study, a longitudinal study examining Merino sheep skin at 1, 3, 6 and 12 months of age could be warranted. As the wool follicle is constantly turning over cells, dramatic changes in telomere length early in life could be life-long consequences on the production of certain types of wool, especially if animals with relatively short telomeres at birth lose large chunks of their telomeres in the first 12 months of life. A well designed study involving sheep with large phenotypic extremes in staple strength and coefficient of variation in fibre diameter would be the best way to test such a theory.

8 Acknowlegements

I wish to thank Don Pegler from Oaklea Genetics for his willingness to participate in this project, and for the great job he did collecting the blood samples from his flock.

The statistical expertise of Associate Professor Wayne Pitchford from the University of Adelaide is also acknowledged. The ASREML analysis on the telomere length data was performed by Wayne and his interest and enthusiasm for this research project was greatly appreciated.

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10 Appendices

10.1 16 digit identification numbers for the sheep used in this study

LPN_ID	LPN_ID	LPN_ID
1500222002020095	1500222005050690	1500992007070761
1500222002020097	1500222005050712	1500992007070789
1500222003030155	1500222005050724	1500992008080053
1500222003030179	1500222005050730	1500992008080067
1500222003030230	1500222005050754	1500992008080073
1500222003030358	1500222005050822	1500992008080081
1500222003030464	1500222005050835	1500992008080128
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