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Running Title: Sex differences in telomere length

Abstract

Mounting evidence suggests that average telomere length reflects previous stress and predicts subsequent survival across vertebrate species. In humans, leukocyte telomere length (LTL) is consistently shorter during adulthood in males than females, although the causes of this sex difference and its generality to other mammals remain unknown. Here we measured LTL in a cross sectional sample of free-living Soay sheep and found shorter telomeres in males than females in later adulthood (>3 years of age), but not in early life. This observation was not related to sex differences in growth or parasite burden, but we did find evidence for reduced LTL associated with increased horn growth in early life in males. Variation in LTL was independent of variation in the proportions of different leukocyte cell types, which are known to differ in telomere length. Our results provide the first evidence of sex differences in LTL from a wild mammal, but longitudinal studies are now required to determine whether telomere attrition rates or selective disappearance are responsible for these observed differences.

Introduction

Telomeres are G-rich repeat sequences of DNA at the ends of chromosomes, which form complexes with proteins that protect linear chromosomes from DNA repair machinery and the so-called 'end replication problem' (Blackburn 1991; De Lange 2004). Telomeres shorten with each cell replication and are also highly sensitive to damage by reactive oxygen species (von Zglinicki 2002), but can be replenished through the action of the enzyme telomerase (Armanios & Blackburn 2012). When telomere length drops below a critical threshold it triggers the onset of cellular senescence, and thus there may be close links between telomere attrition and cellular ageing (Gomes *et al.* 2011; Hemann *et al.* 2001). Average telomere length (TL), which is typically measured from DNA obtained from whole blood samples, has been shown to decline with age in numerous species and is a biomarker of considerable current interest in human epidemiology and evolutionary ecology (Blackburn *et al.* 2015; Monaghan & Haussmann 2006). In humans, TL in adulthood appears predictive of late-onset diseases and, in some studies, mortality risk (Blackburn *et al.* 2015; Cawthon *et al.* 2003; Rode *et al.* 2015), whilst prior experience of chronic stress is associated with shorter TL (Monaghan 2014; Shalev 2012). Studies of wild birds and mammals have also recently found associations between short TL and reduced subsequent survival or recapture rates (Barrett *et al.* 2013; Bize *et al.* 2009; Fairlie *et al.* 2016).

In humans and laboratory rodents, females generally have longer TL than males in adulthood (Barrett & Richardson 2011; Gardner *et al.* 2014). This pattern mirrors the pervasive gender difference in longevity observed in humans and many other mammals, leading to speculation that sex differences in TL may be related to differences in lifespan (Aviv *et al.* 2005; Stindl 2004). A recent meta-analysis

of forty adult human data sets concluded that females have significantly longer telomeres than males (Gardner *et al.* 2014), and more recent large-scale studies have confirmed this pattern (Berglund *et al.* 2016; Lapham *et al.* 2015). However, it remains unclear whether sex differences in TL are present in early life or emerge in later adulthood due to differences in telomere attrition rates in humans. Several studies have failed to document significant sex differences in TL measured in umbilical cord blood (Akkad *et al.* 2006; Aubert *et al.* 2012; Okuda *et al.* 2002; Shi-Ni *et al.* 2013), although a recent larger-scale study did find significantly longer telomeres in new born females than males (Factor-Litvak *et al.* 2016). Studies of captive primates have yielded equivocal results: no sex differences in TL were found in cynomolgus macaques (*Macaca fascicularis*; Gardner *et al.* 2007), whilst female rhesus macaques (*Macaca mulatta*) had significantly longer TL than males in leukocytes but not in other tissue types (Smith *et al.* 2011). In laboratory populations of rats (*Rattus rattus*; Cherif *et al.* 2003; Tarry-Adkins *et al.* 2006) and Algerian mice (*Mus spretus*; Coviello-McLaughlin & Prowse 1997) females were also reported to have longer telomeres than males.. To date, studies of sex differences in TL in non-human mammals remain limited to primates and rodents in captivity: we could find only one study comparing TL between the sexes in a wild mammal population. This study of European badgers, which show strong sexual size dimorphism, found no evidence of sex differences in mean TL or changes in TL with age (*Meles meles*; Beirne *et al.* 2014).

The pattern of sex differences in TL across vertebrate species appears variable, and several non-mutually exclusive explanations have been proposed for this variation, including heterogametic disadvantage and differences in the effects of sex hormones on oxidative stress and telomerase function (Barrett & Richardson 2011; Gardner *et al.* 2014). It has also been hypothesized that, in species with sexual size dimorphism, increased cell proliferation rates and oxidative stress associated with increased growth in the larger sex could reduce TL (Barrett & Richardson 2011; Stindl 2004).

93 Furthermore, increased parasite burdens in males relative to females have been documented in
94 polygynous mammals and proposed as a driver of male-biased mortality (Moore & Wilson 2002).
95 Experiments in laboratory-kept house mice (*Mus musculus*) demonstrated faster leukocyte TL loss in
96 males than females following repeated bacterial infection, raising the possibility that sex differences
97 in TL could reflect sex differences in the response to infection (Ilmonen *et al.* 2008). Polygynous
98 males also typically exhibit secondary sexual characteristics other than increased body size (e.g.
99 seasonal colouration, vocalisations, ornaments and weapons) and highly energetically expensive
100 intra-sexual competition for access to mates (Andersson 1994). Male investment in these
101 reproductive traits may be associated with increased cellular proliferation or oxidative stress,
102 resulting in faster TL attrition in males relative to females (Barrett & Richardson 2011; Monaghan
103 2010). A recent review found little consistent evidence that sex differences in TL were associated
104 with heterogamety, the degree of body size dimorphism, or mating system (Barrett & Richardson
105 2011). However, direct tests for sex-specific associations between TL and either weight, reproductive
106 investment or parasite burden within polygynous species remain rare (Beirne *et al.* 2014; Olsson *et*
107 *al.* 2011).

108

109 In mammals, which have enucleated red blood cells, TL measured in DNA extracted from blood
110 samples reflects the average leukocyte telomere length (LTL). This is in contrast to other vertebrate
111 groups with nucleated erythrocytes, where it is erythrocyte telomere length (ETL) that is
112 predominantly measured in blood. This represents a challenge for mammalian studies, as LTL
113 encompasses a range of different white blood cell types which have different functions and roles
114 within the immune system and show differences in proliferative capacity, telomerase expression and
115 ultimately telomere length (Weng 2001). For instance, in humans and baboons granulocytes have
116 longer telomeres than lymphocytes in adulthood, most likely due to the fact that granulocytes are

117 terminally differentiated cells whilst lymphocytes have the capacity to rapidly replicate and
118 differentiate (Aubert *et al.* 2012; Baerlocher *et al.* 2007; Kimura *et al.* 2010). Lymphocytes also vary in
119 telomere length, with naïve T cells having longer telomere lengths in comparison to memory T cells,
120 again due to greater proliferative history of the latter (Aubert *et al.* 2012; Weng 2001). The
121 composition of circulating leukocyte cell types can change profoundly with age and vary between
122 sexes (Giefing-Kröll *et al.* 2015; Linton & Dorshkind 2004; Pawelec *et al.* 2010), and changes found in
123 average LTL in relation to age and sex could therefore reflect changes in underlying cell population
124 structure (Weng 2001). However, studies in humans and primates have reported very strong within-
125 individual correlations in TL measured in different leukocyte sub-populations and among different
126 tissues (Aubert *et al.* 2012; Baerlocher *et al.* 2007; Daniali *et al.* 2013; Gardner *et al.* 2007; Kimura *et*
127 *al.* 2010). Based on this apparent 'synchrony' in TL across tissue and cell types, it has been argued
128 that among-individual variation in LTL reflects differences in the TL of the haematopoietic stem cell
129 pool, which is primarily determined genetically and by early life environment (Daniali *et al.* 2013).
130 However, studies investigating the dependence of LTL and its associations with age, sex and other
131 traits on variation in leukocyte population structure in non-primate mammals are currently lacking.

132

133 Here, we tested sex differences in LTL in a free-living population of Soay sheep on the St Kilda
134 archipelago. Soay sheep have a polygynous breeding system: males compete for mating
135 opportunities with oestrous females during the autumn rut and have highly skewed reproductive
136 success, with a handful of males obtaining the majority of paternities each year (Clutton-Brock &
137 Pemberton 2004). As is typical in polygynous systems, males are larger and shorter lived than
138 females: by five years of age, males average around 38 kg and females around 24 kg in summer with
139 maximum recorded lifespan of ten years in males and sixteen years in females (Clutton-Brock &
140 Pemberton 2004). This population is parasitized by a variety of Strongyle gastrointestinal nematodes,

and parasite burdens – as estimated by faecal egg counts (FEC) – are predictive of over-winter survival and greater in males than females at all ages (Hayward *et al.* 2009; Wilson *et al.* 2004). Horns, which are grown incrementally each year, are an important secondary sexual trait in males in Soay sheep and horn length is positively associated with subsequent male annual reproductive success (Johnston *et al.* 2013). Both sexes exhibit normal and greatly reduced (scurred or polled) horn growth phenotypes, and the genes underpinning this polymorphism have recently been identified (Johnston *et al.* 2013). We have recently shown that LTL is positively associated with survival in early life in female Soay sheep (Fairlie *et al.* 2016), and that age-related variation in the proportion of different T cell sub-types is present in this population (Nussey *et al.* 2012). However, we have yet to test for sex differences in LTL or examine whether and how LTL is associated with variation in leukocyte cell structure in the population. Here, we examine whether sex differences in LTL are present and whether evident sex differences are dependent on age, weight or Strongyle FEC, as well as testing the hypothesis that costly investment in male horn growth should be reflected by reduced LTL. We also measure the proportions of different leukocyte cell types which are known to differ in telomere dynamics and test whether variation in leukocyte population structure can explain observed associations among LTL, sex, age and investment in secondary sexual characteristics.

Materials & Methods

Study system & sample preparation

The Soay sheep is a primitive breed of domestic sheep that has been living on the remote St Kilda archipelago with minimal human management for the last few millennia. Since 1985, the sheep living in the Village Bay area of the main island in the archipelago, Hirta, have been the subject of

individual-based study (Clutton-Brock & Pemberton 2004). As part of this study, individuals are caught and tagged within a few days of birth in spring. An individual's horn type is identified at capture as follows: "normal" horns are sturdy and consist of a bony core covered in a keratin sheath, whilst "scurred" horns consist of keratin but lack a bony core. A "polled" phenotype is present only in females and involves a complete absence of visible horn growth. Around 85% of males and 35% of females in the population have normal horns (Clutton-Brock & Pemberton 2004). Each August, 50-60% of the resident population are caught in temporary corral traps. At capture, blood and faecal samples are taken and each animal is weighed to the nearest 0.1 kg and horn length is measured from the base of the horn, along the outer curvature to the tip, with each annual growth increment noted (Clutton-Brock & Pemberton 2004). This study uses data and samples from animals caught during the Augusts of 2014 (78 males and 174 females) and 2015 (66 males and 174 females).

Two 9ml Lithium Heparin Vacuettes of blood were taken from each individual and kept in a cool box or fridge from the point of sampling until further processing within 24 hours of sampling. The first Vacuette of blood was spun at 1,008 x g for 10 minutes and the plasma layer was then drawn off and replaced by the same quantity of 0.9 % NaCl solution and spun again at 1,008 x g for 10 minutes. The intermediate buffy coat layer, comprising mainly white blood cells, was then drawn off into a 1.5 ml Eppendorf tube and stored at -20 °C until used to assay leukocyte telomere length. Faecal samples were available for 427 of the captured individuals, and Strongyle and Strongyloides FEC were estimated in these samples using a modified McMaster technique (following Gulland & Fox 1992).

Leukocyte cell measurements

Within 12 hours of collection, 5 µl of whole blood from the second Vacuette was applied to one end of a standard glass microscope slide. The drop of blood was then spread with the edge of a second

slide at a 45° angle to produce an even film. Slides were air dried overnight and stained using a Quick-Diff Kit stain (Gentaur, London) the following day, as per manufacturer's instructions. Differential white blood cell counts were conducted back in the laboratory in Edinburgh. Briefly, 100 cells were counted at 40x magnification using the "battlement track" method and based on staining and morphology, identified as either lymphocytes, eosinophils or neutrophils (Bain 2014). Basophils and monocytes were observed too rarely to analyse. From this data, we calculated the granulocyte (neutrophils and eosinophils) to lymphocyte ratio (GLR) and this ratio was used in subsequent analyses. Only slides with a clear regular monolayer of cells were counted, and slides with uneven cell density or unclear staining were omitted, leaving 465 GLR measurements available for subsequent analyses. See Watson *et al.* (2016) for information on repeatability of these measurements.

A further 1ml of whole blood from the second Vacuette was used to prepare a formalin fixed sample of lymphocytes, which was stored at 4°C until subsequent flow cytometry analysis back in Edinburgh (following Nussey *et al.* 2012). We were able to estimate the proportions of lymphocytes that were helper T cells (CD4+), cytotoxic T cells (CD8+) and, within each of these cell types the proportion that were putatively naïve cells (CD45RA+). Full methodological details are presented in the on-line supplementary material. From this data we calculated the CD4 to CD8 T cell ratio as well as the proportions of helper T cells and cytotoxic cells that were naïve, and we used these three measurements in subsequent analyses.

Telomere length measurement

Genomic DNA was extracted from buffy coat using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's guidelines for animal blood (Cat# 69581, Manchester, UK). The protocol was modified slightly to facilitate sample flow through the spin columns which subsequently improved DNA yield

and purity (see on-line supplementary material). Following DNA extraction and elution in buffer AE (10 mM TrisCl, 0.5 EDTA, pH 9.0), a strict quality control protocol was implemented to determine DNA quality and integrity. First, each sample was individually tested for DNA yield and purity using a Nanodrop ND-1000 9 spectrophotometer (Thermo Scientific, Wilmington DE, USA). Samples yielding < 20 ng/µl were immediately rejected. Samples yielding ≥ 20 ng/µl were checked for DNA purity; acceptable ranges for absorption were 1.7 - 2.0 for 260/280 nm ratio and 1.8 - 2.2 for 260/230 nm ratio. Acceptable samples were then diluted to 10 ng/µl and their DNA integrity assessed by running 20 µl (200 ng total DNA) on a 0.5 % agarose gel. Samples were scored for integrity on a scale of 1 to 5 by visual examination of their DNA crowns, with samples scoring higher than 2 being excluded from further analyses (see Seeker *et al.* 2016 for details). Samples which failed one or more of the above QC measures were re-extracted and if they failed QC a second time they were excluded from the study.

Relative leukocyte telomere length (RTL) was measured using real-time quantitative PCR (qPCR; Cawthon 2002), using protocols we have previously developed and validated in sheep and cattle blood samples (Fairlie *et al.* 2016; Seeker *et al.* 2016). The qPCR method estimates the total amount of telomeric sequence present in a sample relative to the amount of a non-variable copy number reference gene. Note that this method measures both terminal and interstitial telomere sequence, and if interstitial telomeric DNA content varies among or within individuals this could influence our results. In this study we used the beta-2-microglobulin (B2M) as our reference gene, and used primers supplied by Primer Design (Catalogue number: HK-SY-Sh-900, Southampton, UK). For telomeric amplification tel1b (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 were used Epel *et al.* 2004. Telomere primers were manufactured, HPLC purified and supplied by Integrated DNA

Technologies (IDT, Glasgow, UK). Telomere and reference gene reactions were run in separate wells of the same qPCR plate at a concentration of 300 nM and 900 nM respectively. Samples were diluted to 1 ng/µl with buffer AE just prior to qPCR analysis. Each reaction was prepared using 5 µl of LightCycler 480SYBR Green I Master Mix (Cat # 04887352001, Roche, West Sussex, UK) and 1ng of sample DNA in a total reaction volume of 10 µl. We used 384 well plates which were loaded with sample DNA and master mix using an automated liquid handling robot (Freedom Evo-2 150; Tecan).

Each plate included two calibrator samples (1 ng/µl) to account for plate to plate variation and a non-template control (NTC) consisting of nuclease free water. The calibrator sample was extracted from a large quantity of buffy coat prepared from blood supplied from a single domestic sheep (Cat# SHP-BUFCT-LIHP, Sera Laboratories International LTD, West Sussex, UK). We carried out a large number of extractions from this sample, applied the same quality control as above and then pooled the extracts and aliquoted them for subsequent use. Samples, calibrators and NTCs were all run in triplicate. All qPCRs were performed using a Roche LC480 instrument using the following reaction protocol; 10 min at 95 ^{o}C (enzyme activation), followed by 50 cycles of 15 s at 95 ^{o}C (denaturation) and 30 s at 58 ^{o}C (primer annealing), then 30 s at 72 ^{o}C (signal acquisition). Melting curve protocol was 1 min at 95 °C, followed by 30 s at 58 °C, then 0.11 °C/s to 95 °C followed by 10 s at 40 °C.

We used the LinRegPCR software package (version 2016.0; Ruijter *et al.* 2009) to correct our amplification curves for baseline fluorescence, and to calculate well-specific reaction efficiencies and Cq values. A constant fluorescence threshold was set within the window of linearity for each amplicon group, calculated using the average Cq across the first 6 plates. The threshold values used were 0.193 and 0.222, and the average efficiency across all plates were 1.88 and 1.91 for the B2M and telomere amplicon groups, respectively. Samples were excluded from further analysis if the

coefficient of variation (CV) across triplicate Cq values for either amplicon was > 5 %, or if at least one of their triplicate reactions had an efficiency that was 5 % higher or lower than the mean efficiency across all wells on that plate for the respective amplicon. Overall, nine samples were excluded based on quality control failure at either extraction or qPCR stages, leaving 492 samples available for use in further analyses.

Relative LTL for each sample was calculated, following Pfaffl 2001, using average reaction efficiencies for each plate and Cq for each sample determined by LinRegPCR as follows:

$$RTL = (E_{TEL}^{(CqTEL[Calibrator] - CqTEL[Sample])}) / (E_{B2M}^{(CqB2M[Calibrator] - CqB2M[Sample])})$$

Where E_{TEL} and E_{B2M} are the mean reaction efficiencies for the respective amplicon group across all samples on a given plate; CqTEL[Calibrator] and CqB2M[Calibrator] are the average Cqs for the relevant amplicon across all calibrator samples on the plate; and CqTEL[Sample] and CqB2M[Sample] are the average of the triplicate Cqs for the sample for each amplicon.

Data analysis

We began by checking the distribution of our telomere, FEC and leukocyte proportions (GLR, CD4:CD8 ratio, proportion of CD4 and CD8 T cells that were naïve) data. RTL was normally distributed but the other variables showed right skew. Log transformation yielded approximately normally distributions, and so log transformed FEC and leukocyte proportions were used in all analyses that followed. Eleven samples came from animals that were not caught at birth and had uncertain ages, so they were excluded from further analyses. We then calculated the Pearson's correlation coefficient among RTL and the leukocyte proportion measures. To test how RTL varied with age and

sex, we ran linear mixed effect models (LMMs) of RTL including individual identity (481 samples from 395 individuals) and qPCR plate (9 plates) as random effects and year, sex and age (as a linear and quadratic covariate) and the interaction between the age terms and sex as fixed effects. We assessed the significance of each term using likelihood ratio tests (LRTs). We subsequently tested whether independent effects of August weight and FEC on RTL were present, having accounted for age effects, by separately adding weight or FEC and their interactions with sex to the model and testing whether the addition improved model fit using LRTs. We further tested whether horn type was associated with RTL by adding a three way interaction among age, sex and horn type and all associated lower order interactions and sequentially deleting terms until only significant terms remained in the model.

To test our hypothesis that costs of investment in a key secondary sexual trait in males, horn length, might be reflected in RTL, we ran a separate set of LMMs restricting our data set to only normal horned males (N = 130). Only a small proportion of Soay ewes have normal horns and exhibit horn growth (74 out of 348 females in our data set) and this combined with the absence of evidence that horn growth is costly or important in reproduction lead us to restrict our analyses to male horn growth. As horns are grown incrementally each year in sheep, horn length is very strongly determined by an animal's age. To avoid the potentially confounding association between age and horn length in our models (as RTL is age-dependent in males, see Results), we ran a separate models for lambs (aged < 1 year) and adults (aged 1 year or more). In lambs, horn length reflects horn growth over the first four months of life, and we tested its significance by adding both a linear and quadratic horn length fixed effect to a LMM of RTL with plate as random effect (there was only one observation per individual in this data set), alongside year and lamb age in days (as not all lambs were exactly the same age when caught in August) as additional fixed effects. In adult males, horn length inevitably

increases with age as horn increments are grown. We tested the association between horn length and RTL in adults in a similar fashion to lambs, but included age in years as a fixed effect to account for the age-related change in both RTL and horn length.

To test for associations between RTL and leukocyte proportions and determine whether changes in leukocyte cell structure could explain observed patterns of variation in RTL with age and sex, we added all four leukocyte proportion measures into our final overall LMM of RTL and assessed whether previously observed sex and age effects remained significant in this model. We used a similar approach in our models including horn length restricted to normal horned males. For descriptive purposes, we calculated means and standard errors for all immune cell percentages and counts and ratios derived from them both overall and within each major age class (lamb, yearling, adult: 2-6 years, geriatric: >6 years). We then ran LMMs of each immune cell measurement including age, sex and their interaction and year as fixed effects and individual identity as a random effect, and simplified these models as described above. All analyses were conducted in R version 3.2.3 (R Core Team 2012).

Results

There was a significant interaction between the effects of age and sex on RTL ($\chi^2_{(d.f. = 1)} = 5.67$, P = 0.02): males, but not females, showed a decline in telomere length with age (estimated difference in slope between males and females: b = -0.019 +/- 0.008 SE; Figure 1). There was no evidence for significant quadratic effects of age (dropping age^2 and age^2-by-sex interaction from the model: $\chi^2_{(2)} =$ 1.78, P = 0.41) and no significant difference in RTL between capture years (dropping year: $\chi^2_{(1)} = 1.58$,

P = 0.21). When we separated our RTL data set into age groups and re-ran our LMMs (without individual as a random effect, because there were no or very few repeat measures in each data subset), we found that male RTL was only significantly shorter than female RTL in adults aged three years or more (N = 193; males vs. females: b = -0.089 +/- 0.042 SE, $\chi^2_{(1)} = 4.58$, P = 0.03). There was no significant sex difference among lambs (N = 200; b = 0.004 +/- 0.023 SE, $\chi^2_{(1)} = 0.32$, p = 0.86) or individuals aged two years or less (N = 288; b = 0.016 +/-0.020 SE, $\chi^2_{(1)} = 0.68$, P = 0.43). August weight, FEC and horn type were not significantly associated with RTL independent of age and sex. The addition of weight and its interaction with sex to a model already including an interaction between age and sex did not significantly improve model fit ($\chi^2_{(2)} = 1.29$, P = 0.53), and nor did the addition of FEC and its interaction with sex ($\chi^2_{(2)} = 0.36$, P = 0.84). The addition of horn type and its potential interactions with age and sex also did not significantly improve model fit when added to the model including the interaction between age and sex ($\chi^2_{(6)} = 3.12$, P = 0.79).

Horn length was negatively associated with RTL in normal horned male lambs, but not in older males once their age was accounted for (Figure 2). In a model of RTL including only normal horned males, there was a significant decline in RTL with age (b= -0.023 +/- 0.008 SE, $\chi^2_{(1)} = 9.28$, P < 0.01). In a model restricted to lambs only, there was no effect of the lamb's age in days at capture in August ($\chi^2_{(1)} = 0.35$, P = 0.55) and a quadratic effect of horn length was not significant ($\chi^2_{(1)} = 0.62$, P = 0.43). Horn length was significantly negatively associated with RTL (N = 88; b= -0.0010 +/- 0.0004 SE, $\chi^2_{(1)} =$ 5.43, P < 0.05; Figure 2A). This association remained significant, and actually became stronger, when lamb August weight was included in the model (b= -0.0018 +/- 0.0005 SE, $\chi^2_{(1)} = 11.56$, P < 0.001). In models including adult normal horned males, horn length was significant when age was not included in the model (N = 42; b= -0.0007 +/- 0.0002 SE, $\chi^2_{(1)} = 10.06$, P < 0.01; Figure 2B), but was non-

significant when the age was included in the model (b= -0.0002 +/- 0.0003 SE, $\chi^2_{(1)} = 0.59$, P = 0.44; Figure 2C).

Means and standard errors for all immune cell measurements are presented in Table S2. All four measures of leukocyte proportions used in analyses (GLR, CD4:CD8 ratio and the proportion of naïve CD4 and CD8 T cells) were significantly associated with age: GLR increased with age and the other three measures declined (Figure 3, Table S3). GLR and the proportion of CD4+ naïve T cells showed significant age by sex interactions: males increased their GLR and decreased their proportion of CD4 naïve T cells more rapidly with age than females (Figure 3, Table S3). Variation in RTL and its association with sex and age were largely independent of variation in the proportions of different leukocyte cell types measured in the samples. Pearson's correlation coefficients among RTL and the ratios and proportions of leukocyte cell types ranged between +0.1 and -0.1 (Figure 4). In a LMM of RTL restricted to samples for which all leukocyte proportion measures were available (N = 437), the age by sex interaction remained marginally non-significant (male vs. female slope: b = -0.016 +/- 0.009 SE, $\chi^2_{(1)} = 3.46$, P = 0.06). Addition of all four leukocyte proportion measurements to the model of RTL did not improve model fit ($\chi^2_{(4)} = 3.34$, P = 0.50) or meaningfully alter the effect sizes or significance of the age by sex interaction (b = -0.015 +/- 0.009 SE, $\chi^2_{(1)} = 3.16$, P = 0.08). The significant negative association between RTL and horn length in male lambs remained when all four leukocyte cell type measurements were included in the LMM (N = 80; b = -0.0010 +/- 0.0005, $\chi^2_{(1)} = 4.23$, P < 0.05) and, as before, the addition of the four leukocyte measurements to the model did not improve fit ($\chi^2_{(4)} = 4.18$, P = 0.38).

Discussion

This study provides, to our knowledge, the first evidence for sex differences in telomere length from a wild mammal. Differences in LTL between males and females were not detectable before three years of age, suggesting that there was no sex difference in LTL at birth in our study system. Longer LTLs in females than males in adulthood but not early life have also been documented in humans and laboratory rodents, suggesting that these sex differences in LTL may arise as a result of differences in attrition rates through development and early adulthood (Cherif *et al.* 2003; Gardner *et al.* 2014; Lapham *et al.* 2015; Tarry-Adkins *et al.* 2006). In our study, the presence of shorter LTL in older males compared to females could be due to sex differences in telomere attrition rate or in selective mortality associated with telomere length. Sex differences in selection on erythrocyte telomere length have been documented in wild sand lizards (*Lacerta agilis*; Olsson *et al.* 2011), and winter mortality in Soay sheep on St Kilda is male-biased at all ages (Clutton-Brock & Pemberton 2004). Current evidence from wild vertebrates, including a previous study of Soay sheep, points to positive associations among LTL or ELT and either annual survival or longevity and thus selective disappearance of individuals with short telomeres (Barrett *et al.* 2013; Beirne *et al.* 2014; Bize *et al.* 2009; Fairlie *et al.* 2016; Olsson *et al.* 2011; Salomons *et al.* 2009). Although sex differences in telomere attrition rate could explain our results, the presence of stronger selective disappearance of individuals with short telomeres in females than males could also be responsible. We had insufficient longitudinal repeat samples within our very largely cross-sectional data set to differentiate these two possibilities. Longitudinal telomere data from both sexes spanning the period from birth to later adulthood are required to fully understand the within- and among-individual processes responsible for sex differences in telomere length.

We found no association between LTL and weight or Strongyle FEC in either sex, suggesting sex differences in growth or body size and in infection with gastrointestinal parasites could not explain observed sex differences in LTL in later life. This adds to the general lack of support for the sexual size dimorphism hypothesis from both among- and within-species studies (Barrett & Richardson 2011; Beirne *et al.* 2014; Olsson *et al.* 2011). Previous studies have found associations between micro-parasite infection status and telomere length (Asghar *et al.* 2015; Asghar *et al.* 2016; Ilmonen *et al.* 2008), whilst our study measured burden with chronically infecting gastro-intestinal nematode parasites. It seems plausible that a larger longitudinal study may be required to detect the immune consequences of such highly localised and long lasting infections and telomere lengths. Studies in laboratory rats have suggested sex differences in TL emerge around puberty as a direct result of the differential effects of sex hormones on telomere dynamics (Cherif *et al.* 2003; Tarry-Adkins *et al.* 2006). Soay sheep are sexually mature in their first year, so our data imply that sex differences in LTL emerge several years after puberty, but could still be the result of cumulative telomere eroding effects of testosterone relative to oestrogen. Over and above hormonal causes, males surviving to later adulthood will have experienced the cumulative physiological demands associated with years of rutting which could generate further differences in the rate of telomere attrition compared to females.

We have presented rare evidence of reduced telomere length associated with investment in a reproductive trait under natural conditions. Studies of birds in both laboratory and wild populations have found that experimentally increasing reproductive effort decreased ETL in parents, in the short term at least (Heidinger *et al.* 2012; Reichert *et al.* 2014; Sudyka *et al.* 2014, but see Beaulieu *et al.* 2011; Voillemot *et al.* 2012), whilst a non-manipulative field study found negative associations between ETL and arrival date and the number of nestlings (Bauch *et al.* 2013). We found a significant

427 negative association between LTL and horn length in males at around four months of age, but not in
428 adult males. This is consistent with LTL shortening reflecting some physiological cost of horn growth,
429 but raises the question of why it was only detected in lambs. Lambs aged four months are growing
430 rapidly and contending with their first exposures to the parasite fauna on St Kilda (Clutton-Brock &
431 Pemberton 2004), and this may mean that the costs of investment in secondary sexual traits such as
432 horn growth are most pronounced at this age. This stage also captures the relationship between LTL
433 and horn growth prior to potentially confounding effects of over-winter viability selection and
434 subsequent rutting effort and incremental horn growth, which could make the cost easier to detect.
435 A study of wild sand lizards documented disruptive selection on ETL in females but not in males
436 (Olsson *et al.* 2011), and previous work on Soay sheep showed that the alleles associated with horn
437 growth improves breeding success at a cost to longevity in males (Johnston *et al.* 2013). These
438 studies and our present results suggest that the degree to which sex differences in LTL are generated
439 and maintained by sexual differences in selection on telomere length is an important area for future
440 study.
441
442 Our study offers indirect evidence that the observed relationships among LTL, age and sex are not
443 driven by variation in the proportions of particular leukocyte cell types, which are known to have
444 different telomere lengths during adulthood in humans (Aubert *et al.* 2012; Kimura *et al.* 2010; Weng
445 2001). We documented age-related changes in the proportions of different leukocyte cell types that
446 were consistent with observations in humans and laboratory mice, and in our own previous studies
447 of this system (Linton & Dorshkind 2004; Nussey *et al.* 2012; Pawelec *et al.* 2010). Of particular note,
448 the decline in the proportion of naïve helper and cytotoxic T cells (Figure 3) with age could generate
449 population level declines in mean LTL because naïve T cells have longer telomeres than effector and
450 memory T cells (Aubert *et al.* 2012; Weng 2001). Furthermore, the presence of a sex by age

interaction for CD4+ naïve cells, indicating that males have more rapid rates of declines with age than females (Figure 3, Table S3), could have been responsible for the observed sex difference in LTL with age. However, we found little evidence that LTL was correlated with any of the leukocyte proportion measurements and we showed that the main results of our LTL models were not influenced by the inclusion of the leukocyte proportion measurements. Our remote field study site precluded the use of more sophisticated methods to determine the actual telomere lengths of particular types of leukocytes, such as cell sorting or flow-FISH (Kimura *et al.* 2010; Aubert *et al.* 2016). However, our findings are consistent with the mounting evidence that TL is highly correlated across leukocyte cell subsets, and across tissues more generally, within individual organisms (Asghar *et al.* 2016; Aubert *et al.* 2012; Daniali *et al.* 2013; Kimura *et al.* 2010; Reichert *et al.* 2013). This suggests that telomere length, whether measured in leukocytes or erythrocytes, could reflect variation that exists in the individual's haematopoietic stem cell compartment, and even more general organism-wide variation in telomere length (Daniali *et al.* 2013; Kimura *et al.* 2010; Reichert *et al.* 2013).

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623 JM), pp. 113-165. Cambridge University Press, Cambridge.

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626 **Data accessibility:** Data used in this paper is available on Dryad:
627 http://dx.doi.org/10.5061/dryad.kd92s.

628

629 **Authors' contributions:** All authors contributed to the design of the study; RLW and DHN
630 wrote the manuscript with editorial input from all co-authors; RLW, HF and DHN conducted the
631 statistical analyses; RLW, EJB, JF, SU, KW, ES-C and RVA conducted the laboratory work; JMP and JGP
632 coordinated field trips and conducted data and sample collection in the field.

Figure 1. Older males have shorter relative leukocyte telomere length (RTL) than females, but no sex differences are apparent amongst young animals. Raw data for males (blue triangles) and females (red circles) are presented with a linear regression (blue and red lines respectively) and associated standard error (grey shading) for each sex.

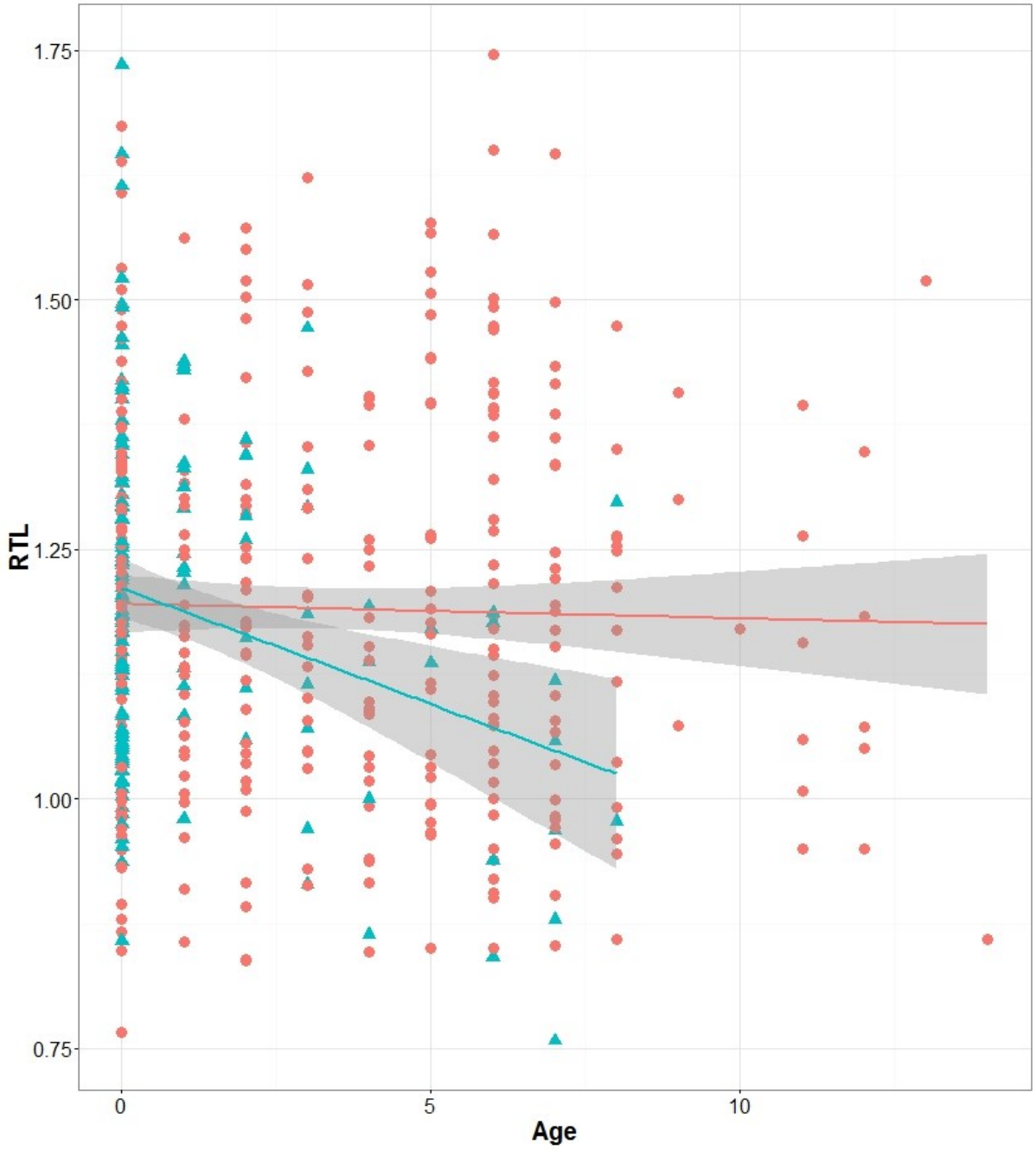

Figure 2. Relative leukocyte telomere length (RTL) is negatively associated with horn length amongst normal horned males in lambs (<1 year old), but not adults (1 year old or greater) once effects of age are accounted for. Plots show raw RTL against horn length measures with linear regression (black line) and associated standard error (grey shading) for: (A) male lamb, (B) adult males, (C) adult males, having corrected horn length for age by taking residuals from a regression of RTL on age.

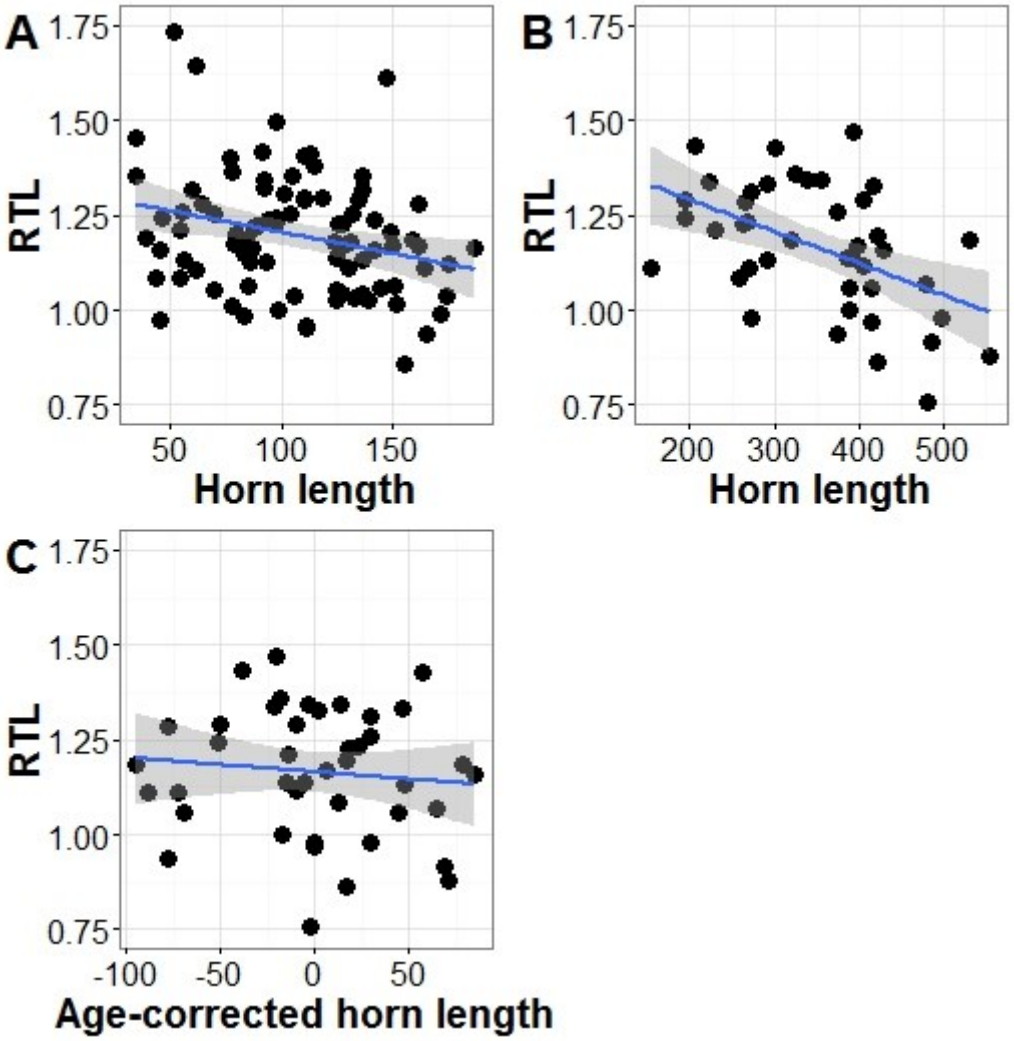

Figure 3. Scatter plots showing relationships with age and sex for (A): granulocyte to lymphocyte ratios, (B): CD4 to CD8 T cell ratios, (C) the proportion of CD4+ "helper" T cells that were naïve and (D) the proportion of CD8+ "cytotoxic" T cells that were naïve. All proportions and ratios are log transformed. Raw data for males (blue triangles) and females (red circles) are presented with a linear regression (blue and red lines) and associated standard error (grey shading) for each sex.

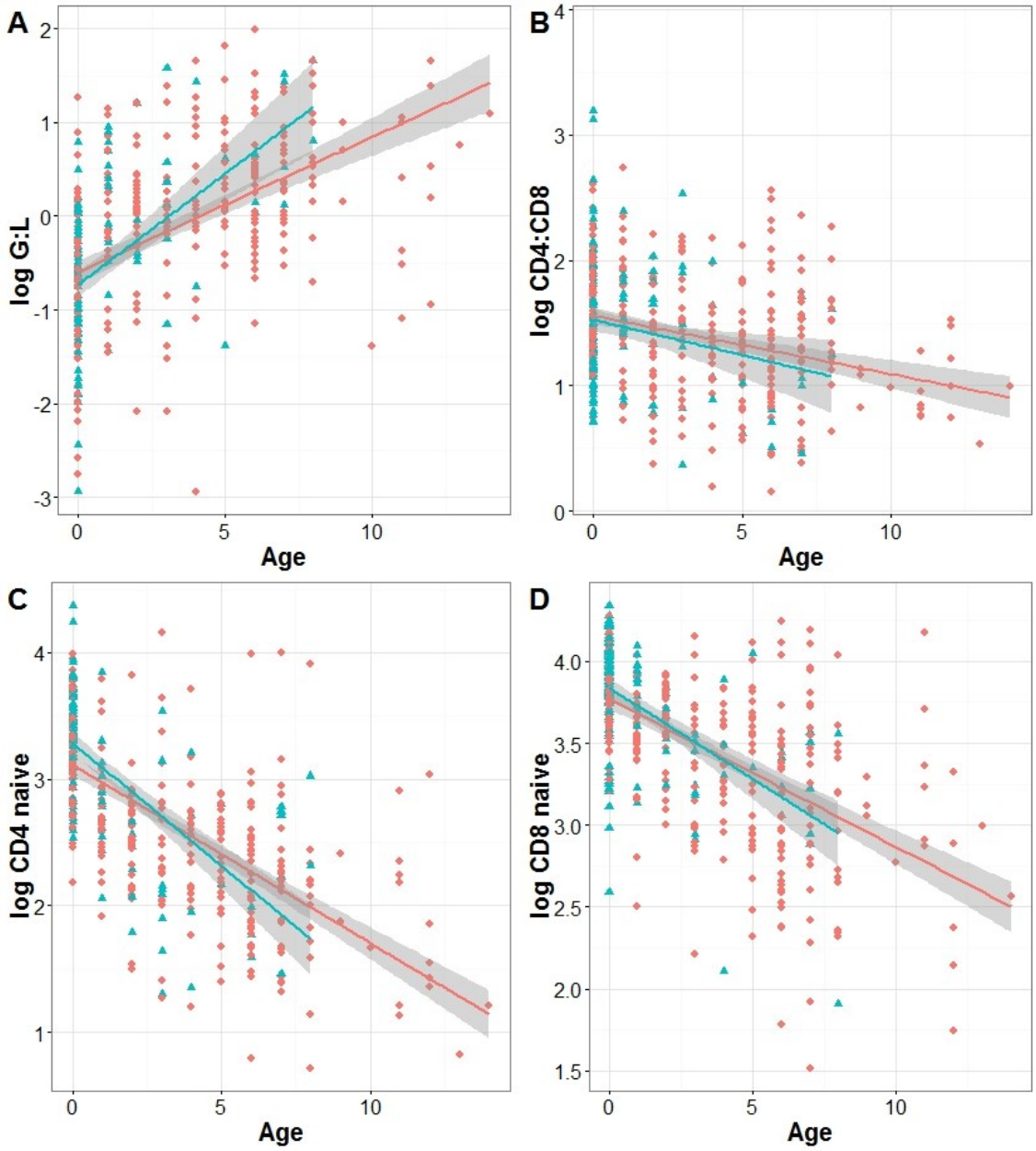

Figure 4. Scatter plots illustrating correlations among relative leukocyte telomere length (RTL) and measures of the proportion of different leukocyte cell types. Scatterplots show relationships between RTL and: (A) granulocyte to lymphocyte ratio ("G:L ratio"), (B) CD4+ T cell to CD8+ T cell ratio ("CD4:CD8 ratio"), (C) proportion of CD4+ T cells positive for CD45RA ("CD4 naïve") (D) proportion of CD8+ T cells positive for CD45RA ("CD8 naïve"). All leukocyte ratios and proportions were log transformed; Pearson's correlation coefficients and associated P values presented for each plot.