Sperm telomere dynamics in a small songbird, the blue tit (*Cyanistes caeruleus*)

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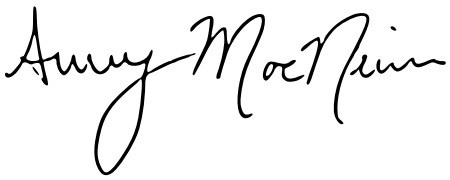

-AVERY MACNEISH

Abstract

There is much interest in research on sperm telomere dynamics and how telomere length relates to sperm quality, male fertility, and its impact on fitness. There is, however, a lack of knowledge on sperm telomeres in avian species. This thesis aims to explore sperm relative telomere length in relation to blood telomere length, age, and individual attributes of adult male blue tits (*Cyanistes caeruleus*) in a wild population, using both cross-sectional and longitudinal data. Fieldwork was carried out in Dæli, outside of Oslo, Norway. A qPCR assay was used to estimate relative telomere length in 50 sperm samples and 40 blood samples from 45 individuals collected in 2016 and 2017, and correlations between relative telomere length in sperm and various attributes were assessed. There was a significant positive correlation with blood relative telomere length and a significant year effect on sperm telomere length. Cross-sectional data indicated that there was no relationship between sperm telomere length and age in blue tits; however, longitudinal data revealed an increase in sperm telomere length for all five individuals analysed. In addition, males with longer sperm telomeres reared broods which had lower mean nestling masses in one of the study years. The results indicate that sperm telomere dynamics are similar to those of blood telomeres in the short-lived blue tit, in contrast to what has been found in humans and other mammals.

Contents

1 Introduction

Since the mid-19th century, the characteristics of spermatozoa have been of interest when exploring fertility (Mantegazza, 1866). Much research has since been put into exploring how sperm count, motility, and morphology play a role in sperm quality and fertility, in both humans (MacLeod & Gold, 1951; Bonde *et al*., 1998; Guzick *et al*., 2001; Nallella *et al*., 2006) and other animals (Birkhead *et al*., 2008). Sperm DNA integrity has also been found to be correlated with fertility and has been suggested to be a better predictor of male fertility potential than the standard sperm parameters mentioned above (Lopes *et al*., 1998; Lewis & Aitken, 2005; Aitken *et al*., 2010).

In recent years, the role of telomerase in germ cells and telomere length in mature sperm cells have been of interest when examining fertility and sperm quality (Thilagavathi *et al*., 2013a, 2013b; Ferlin *et al*., 2013; Liu *et al*., 2015; Vecoli *et al*., 2017), maintenance of the male germline (Pech *et al*., 2015), and how paternal age and telomere length contributes to the viability of future progeny (Nordfjäll *et al*., 2005; Njajou *et al*., 2007; Kimura *et al*., 2008; Nordfjäll *et al*., 2010).

Telomeres are non-coding DNA sequences, consisting of short, tandem repeats of nucleotides, often rich in guanine (G) which, together with shelterin proteins, form caps at the ends of chromosomes in eukaryotes. The number of repetitions of this sequence (TTAGGG in vertebrates) is referred to as the length of a telomere and varies among species. They function to provide chromosomal stability by preventing (1) chromosomal end-to-end fusion, to itself or other chromosomes, and (2) degradation of the terminal coding regions of chromosomes through incomplete replication of the 5' strand, also known as the "end-replication problem" (Blackburn, 1991; Blackburn, 2005; Heidinger *et al*., 2012). Telomeres provide protection to the important coding region of DNA by acting as a buffer between it and the terminal degradation during replication, which is reflected by the shortening of telomeres with organismal aging (Aubert & Lansdorp, 2008; Haussmann *et al*., 2007). Once telomeres reach a critically shortened length, the cell enters a final non-dividing state known as cellular/replicative senescence (Hornsby, 2002).

Cellular division is not the only factor responsible for telomere shortening. Telomeres have been found to be highly sensitive to different stress factors such as oxidative stress, alkylation, and ultraviolet (UV) radiation. These stresses can contribute to a greater loss of telomeric

nucleotides than through normal loss during replication as they induce single and double-strand breaks in telomeres (von Zglinicki, 2002). Due to the vulnerable nature of telomeres to oxidative stress, they are said to act as wardens for the entire genome, giving indications that there is likely to be DNA damage in coding sequences, without having to screen the entire genome. Instead, telomere loss due to oxidative damage may trigger senescence as a response to mutation risk (von Zglinicki, 2003).

As shown above, there are many elements (including others not stated) that play a role in the reduction of telomere length. However, it has been established that so-called immortal cells and most cancer-derived cell lines exhibit no net loss of telomere length with cell division (Kim *et al*., 1994). For this, there must be some way to counteract this loss and maintain telomere length. Telomerase is a ribonucleoprotein enzyme that elongates telomeres by synthesizing telomeric DNA repeats onto chromosomal ends. Using its own RNA template, it catalyzes the addition of nucleotides *de novo* (Greider, 1996; Kim *et al*., 1994). Telomerase is most notably expressed in most cancer cell and immortal cell lines, as well as the germ line and early embryonic cells. In cancer and immortal cells, telomerase activity is a means of maintaining telomere stability and evading replicative senescence. The majority of human adult somatic cells exhibit repressed telomerase activity and undergo telomeric shortening with increasing age (Campisi, 2001; Forsyth *et al*., 2002; Kim *et al*., 1994). It is postulated that repression of telomerase in somatic cells and the role of replicative senescence evolved to inhibit the growth of cancer cells (Blackburn *et al*., 2006)

Telomerase plays an important role in germline cells that give rise to mature spermatozoa, which do not display telomerase activity (Wright *et al*., 1996; Fradiani *et al*., 2004; Pech *et al*., 2015). High expression of telomerase in the testes is thought to be due to the continual need for sperm production throughout life (Asghar *et al*., 2015). In humans, while telomere length shortens in somatic cells, sperm telomere length is known to increase with age (Allsopp *et al*., 1992; Baird *et al*., 2006; Benetos *et al*., 2001; Kimura *et al*., 2008; Aston *et al*., 2012) and longer telomere length in sperm of older males may be regarded as an advantageous effect of increased paternal age on the prospects of progeny (Ferlin *et al*., 2013). Telomerase activity in the germline is important for ensuring that gametes and subsequent progeny will be able to contribute to future generations as complete inactivation of telomerase in model organisms, such as roundworms, mice, and fish, causes infertility and the eventual loss of male germ cells (Lee *et al*., 1998; Meier *et al*., 2006¸ Henriques *et al*.,

2017). It has been hypothesized that short telomeres in the germline may impede spermatogenesis via segregation errors and apoptosis of germline cells resulting in a reduced sperm count (Ferlin *et al*., 2013). In mice and several avian species of varying lifespans, telomerase activity was elevated in gonadal tissues at all stages of life (Riou *et al*., 2005; Haussmann *et al*., 2007). Studies on humans make up the majority of research on sperm telomeres. However, to my knowledge, no studies have so far been published on avian sperm telomere length dynamics, which are the focus of this thesis.

The Eurasian blue tit, *Cyanistes caeruleus* (hereafter, blue tit), is a small, short-lived passerine that is frequently used in ecological and evolutionary studies. Blue tits are abundant in the Oslo, Norway area and are accessible for studying due to their use of provided nest boxes in the study site. They exhibit a high tolerance for disturbance, making them an appropriate candidate for handling and sperm and blood sampling. Several studies have been published on telomere dynamics in somatic (blood) cells of blue tits. Sudyka *et al*. (2014) revealed that adults with experimentally increased brood sizes experienced more yearly telomere erosion than control birds, and in a 2016 study (Sudyka *et al*., 2016), they reported that blue tits had one of the highest rates of telomere shorting in a comparison of long- and short-lived birds. Furthermore, a study by Badás *et al*. (2015) showed that antioxidant supplementation to blue tits reduced telomere loss within a year of treatment.

This thesis will explore telomere dynamics in sperm of individual blue tits and how sperm telomere length relates to blood telomere length, year, and age. Based on results obtained in human studies (Allsopp *et al*., 1992; Baird *et al*., 2006; Kimura *et al*., 2008; Aston *et al*., 2012; Ferlin *et al*., 2013), I predict that there will be a positive correlation between sperm telomere length and blood telomere length, and that sperm telomere length will increase with male age. In addition, I will examine whether telomere length in sperm is related to rearing conditions, estimated by brood size as a chick, and male morphology (body mass, tarsus length, and wing length) as an adult. Lastly, I will investigate telomere length in sperm in relation to different measures of reproductive success, estimated by laying date, clutch and brood size, number of fledglings, and mean nestling mass.

2 Materials and Methods

2.1 Study area and focal species

Between the end of March and end of June 2017, field work was carried out at Dæli ($59^0$56'N, $10^0$32'E), located approximately 10 kilometers west of Oslo, Norway. This area is a rich woodland habitat composed of primarily deciduous trees scattered with conifers. Within, there lie several small settlements, farmland, a cemetery, and the 1.6 km^2 study area which has been used since 1995 for various studies pertaining to passerine birds. Roughly 550 wooden nest boxes are available for nesting each year and are dispersed 40 – 50 m apart throughout the study area. They are fastened to the trunks of trees at ~1.5 m above the ground for ease of inspection.

Blue tits are small passerine birds that are easily recognized by their blue and yellow plumage. To the human eye they are sexually monochromatic (see Anderson *et al*., 1998). Adults typically range from 10-12 g in weight, with males being slightly larger than females. They inhabit cavities in trees, and nest boxes when available, where they use moss, straw, feathers, hair, and wool collected from the ground as nesting material. Females are responsible for nest building and incubation, and lay about 4 – 14 eggs, while the male aids in feeding her during the incubation period of approximately 13 – 16 days. Once hatched, the nestlings are fed by both parents until ready to fledge after 16 – 22 days. A blue tit's diet during the summer consists primarily of insect larvae, flies, and spiders, whilst through the winter they rely on seeds and berries (Haftorn, 1971).

From 1995, the local populations of blue tits and other passerine species such as the great tit (*Parus major*), the pied flycatcher (*Ficedula hypoleuca*), the Eurasian nuthatch (*Sitta europaea*), and the coal tit (*Periparus ater*) found using nest boxes in Dæli have had thorough yearly monitoring, which has provided detailed life histories of the individuals. In the spring of 2017, 120 blue tits were found nesting in the provided boxes, in addition to 64 great tits, 17 nuthatches, 49 pied flycatchers and a few coal tits. Blue tits have an adult survival rate of about 46% and approximately 5 – 10% of the fledgling cohort returns to the area as local recruits the following year (Slagsvold *et al*., 2002).

2.2 Data collection

At the end of March, nest boxes were cleaned of old nesting material and fecal matter to remove the majority of ectoparasites and prepare for the new breeding season. The boxes were then inspected every 2 – 3 days to observe signs of early nest building and follow nest development. Ownership of occupied boxes was also determined during this period through observation of individual colourband combinations. In this way survival of birds sampled in 2016 could be estimated. Nests were continuously checked post nest building to acquire accurate egg laying and hatching dates. Females generally lay one egg per day early in the morning (Haftorn, 1971); this was assumed when estimating date of first egg. During the incubation period nest visitation was ceased until the estimated date of hatching in order to not disturb the females. Within 0 – 3 days after hatching, nestlings were counted and weighed with a Pesola 10 g spring balance to deduce hatching date of the first nestling from a growth curve. At approximately 15 days old the nestlings were banded with an aluminum band containing a unique identification number and re-weighed. Additionally, blood samples (5 - 25µl) taken from the brachial vein were attained from nestlings belonging to parents that had been captured that same season. Blood samples were deposited into 2ml microtubes containing 1ml 96% ethanol but, were not used in this study. Post fledging, nest boxes were re-visited to see how many nestlings did not survive and to determine the number of chicks fledged. The body mass of those nestlings that successfully fledged was used to obtain a mean nestling mass for each brood.

Adults were captured close to their nest boxes using a mist net, male song playback, and a captive male from another study area. Clap traps in nest boxes were also used to catch several females and a few males. This was done during the period of May 5th to June 13, when most females were incubating or brooding. 25 of the 48 blue tits that were captured were local recruits of known age and 23 were immigrants. Many of these immigrants had been captured prior to the spring of 2017, mostly at feeding stations in fall 2016. Immigrant ages were assessed from plumage colour and deemed “first year” or “older”, according to Svensson (1992). Males were prioritized in the capturing of adults due to a focus on collecting sperm samples, as were individuals from both sexes who had been captured and sampled in the spring of 2016. Local recruits were also of high interest due to the available information on their life histories and rearing conditions. Individuals from 41 nest boxes were captured, comprising 34% of all nest boxes housing blue tits in the study area in 2017.

Once captured, unbanded immigrants were fitted with a unique combination of three colour bands and one aluminum band containing a unique identification number. Body mass, tarsus length, and wing length were recorded using a 50 g spring scale, a caliper, and a ruler with a zero stop, and blood samples (5-25µl) were taken from the brachial vein and deposited in 2ml microtubes containing 1ml Invitrogen RNAlater® Stabilization Solution (Thermo Fisher Scientific). Sperm samples (1-3µl) were taken by cloacal massage (Wolfson, 1952) and a small fraction was deposited in 2ml microtubes containing 1ml 5% formaldehyde and the larger fraction in 2ml microtube containing 1ml 96% ethanol to be used for later DNA analyses. For confident identification, each individual and their band combinations were photographed and within an hour of capturing, all birds were released in the vicinity of their nest boxes.

It is common to use blood samples in analyses of avian telomeres, as avian erythrocytes (red blood cells) contain nuclear DNA and only a small, easily-obtained sample of blood is required. It was found in zebra finches (*Taeniopygia guttata*) that the telomere length in red blood cells is related to the telomere length in other somatic tissues. It is assumed that this relation is consistent across avian species (Reichert *et al*., 2013), which is why blood will be used in this study in conjunction with sperm.

All collected samples were registered in the DNA bank database Corema (Collection and research Management) at the Natural History Museum, University of Oslo. Blood and sperm samples were stored in a refrigerator (4°C) up until and during laboratory analysis at the museum, where they were later stored in a -80°C freezer in the DNA bank.

2.3 Ethical note

Nest visitation was minimized as much as possible, especially during incubation, and each nest was never disturbed for more than an hour at a time. Nests were not visited during periods of cold weather or snowfall. Caution was observed when taking blood samples so as to not take more than was needed for analysis (<25µl). Handling of birds and blood sampling seemed neither to have an effect on survival nor fledging success of nestlings (personal observation). Authorization for blood collection and temporarily caging males was granted by the Norwegian Food Safety Authority (Mattilsynet), and authorization for banding and the use of mist nets was given by the Norwegian Environment Agency (Miljødirektoratet).

2.4 Data set

This master's thesis was done in conjunction with Kristiane Hol, who completed her master's investigating telomeres in blood of females and males (Hol, 2018). Both studies are a continuation of work completed by Ingvild Aabye (Aabye, 2017). The samples collected and analysed by Ingvild Aabye in 2016 were also included in this study, in order to increase sample size and explore longitudinal trends of telomere dynamics. Thus, the laboratory protocol used by Inglvild Aabye was also followed for the present study. Sperm samples from 2016 had not yet been analysed and due to the sensitivity and inter-observer dependent nature of the Quantitative Real-time Polymerase Chain Reaction (qPCR) method, blood samples from 2016 were re-analysed in 2017 to allow comparisons with samples collected in 2017. All field work and laboratory work was shared with Kristiane Hol and a total of 162 blood and sperm samples were processed. The final data set used for the purpose of this thesis includes 50 sperm samples and 40 blood samples from 45 adult males. Of these, 26 sperm samples and 23 blood samples were from 2016, and 24 sperm samples and 17 blood samples were from 2017. The blood samples used in this study were those that corresponded to a sperm sample from the same individual. Five individuals were caught and sampled in both 2016 and 2017.

2.5 Laboratory work

2.5.1 Blood DNA extraction

Extraction of DNA from blood samples was done using the commercially available E.Z.N.A® Blood and Tissue DNA Kits (Omega bio-tek). Standard protocol was followed with some changes: Once thawed, 50µl of each sample was mixed with 150µl of elution buffer, 20µl of Proteinase K (OB Protease Solution), and 200µl of BL buffer, and vortexed to mix thoroughly. This mixture was incubated at 70°C for 30 minutes, briefly vortexing the tube midway through to ensure cells lysis. 200µl of absolute ethanol was added and vortexed to precipitate the DNA and decrease solubility. The precipitated DNA was filtrated using a HiBind DNA mini column and washed with 400µl of HBC buffer. When ready for elution, 100µl of elution buffer heated to 70°C was added to the HiBind DNA Mini Column and was allowed to rest for 20 minutes before centrifugation into a final 1.5ml microcentrifuge tube. DNA concentration of each sample was measured using the Invitrogen Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). Concentrations of DNA ranged from 1.32ng/µl to 67.3ng/µl. Extracted DNA samples were stored in a refrigerator at 4°C until qPCR analysis.

2.5.2 Sperm DNA extraction

Sperm samples underwent a preliminary wash prior to extraction, using a modified method from Griffin (2013), to wash away seminal fluids and any somatic cells that may have been in the sample. Sperm were washed in two volumes (200µl) of sperm wash buffer (150mM NaCl, 10mM EDTA, pH 8.0) and centrifuged at 750 x *g* for 10 minutes after each wash. The supernatant was poured off after first wash and cells resuspended by vortexing upon addition of the second wash. The supernatant was then poured off carefully to avoid agitating the sperm pellet.

Extraction of DNA from sperm samples was done using the protocol for Isolation of Genomic DNA from Forensic Case Work Samples from the QIAamp® DNA Micro Kit 08/2003 (QIAGEN). Standard protocol was followed with minor changes: Before final elution of the DNA sample, 20µl of elution buffer was added to the QIAamp MinElute Column and allowed to incubate at room temperature for 15 minutes before being centrifuged. The concentration of DNA of each sample was measured using the Invitrogen Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) and concentrations ranged from 0.73ng/µl to 42.5ng/µl. Extracted DNA samples were stored in a refrigerator at 4°C until qPCR analysis.

2.5.3 Estimating the T/S-ratio with qPCR

To obtain an estimate of relative telomere length, qPCR was used. The protocol has been optimized for blue tit samples by Angela Pauliny at the University of Gothenburg, based on a protocol developed by Cawthon (2002). qPCR enables the monitoring of PCR product amplification in real time with the use of a fluorescent double stranded DNA-binding dye (SYBR® Green). In principle, the amount of product doubles with each PCR cycle, accumulating exponentially until all reaction components are used. The qPCR instrument will detect an emitted fluorescent signal when the amount of PCR product reaches a threshold. The cycle for which this occurs is known as the quantification cycle (Cq).

Estimating relative telomere length requires qPCR to obtain a telomere to single-copy reference gene ratio (T/S-ratio) in a given sample. The T/S-ratio is the number of telomeric repeats (T) relative to the amount of a single-copy reference gene (S), in this study being the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The T/S-ratio represents the mean length of telomeres in all cells within a given sample, for every cell should only possess a single copy of the reference gene.

The Cq-vaule is used in calculating a telomere to single-copy reference gene ratio (T/S-ratio) in a given sample, or relative telomere length.

Overview of qPCR protocol

qPCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Samples were run on clear-well plates (Hard-Shell® 96-Well PCR Plates, Bio-Rad) covered with clear adhesive seals (Microseal® 'B' Adhesive Seals, Bio-Rad). The real-time PCR reagent SSoAdvanced^{TM} Universal SYBR® Green Supermix (Bio-Rad) was used in all assays. Program details used for the amplification of telomeres and the reference GAPDH gene are depicted in Table 1. Because the qPCR assay is especially sensitive to differences in concentrations of DNA and SYBR®Green Supermix, there was extra vigilance in the pipetting technique to ensure this did not vary between wells or plates. Assays of all 162 samples were performed in a total of 10 separate batches over a two month period. Ideally the analysis of multiple plates should be accomplished in as little time as possible to maintain consistent pipetting techniques, but this was not possible due to schedule constraints. Procedures were kept within the scope of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009) to ensure reliability, quality, and repeatability of all results.

Table 1. qPCR programs for telomere- and GAPDH-amplification of blue tit DNA

	Telomeres	GAPDH
Denaturation	96°C, 3 min	96°C, 3 min
Amplification	96°C, 15 sec → 56°C, 45 sec (x25)	96°C, 15 sec → 60°C, 45 sec (x40)
Melting Curve	55-96°C (0.5°C increase/cycle), 5 sec	59-96°C (0.5°C increase/cycle), 5 sec
Hold	15°C	15°C

Primers used for the amplification of telomeres were Tel1b (forward) and Tel2b (reverse) as published by Criscuolo *et al*., (2009). The reference GAPDH gene was amplified with the forward primer GAPDH-F and the reverse primer GAPDH-R (Criscuolo *et al*., 2009). These GAPDH primers are specific to the zebra finch GAPDH gene, but were studied and proven useful for blue tit DNA by Angela Pauliny.

Assessment of amplification efficiency

Standard curves for telomeres and the reference GAPDH gene, in both blood and sperm samples were created to assess the efficiency of the qPCR assay (Svec *et al*., 2015). Standard curves were produced 1-2 days before the first plates of samples were analysed, and again after the tenth plate. One DNA sample (of each blood and sperm) was used to create separate 45µl stock concentrations of 1ng/µl for telomeres and 2ng/µl for GAPDH to be used for a serial dilution (1:3) with six technical replicates with six duplicates at each concentration (see appendix 1). All wells had a total volume of 10µl, containing 4µl serial diluted DNA and 6µl of a master mix containing 5µl 1X SYBR® Green Supermix and 0.5µl of both forward and reverse primers per well (3.5µM for both forward and reverse GAPDH primers; 2µM for the Tel1b forward primer and 4µM for the Tel2b reverse primer). A master mix was prepared separately for each standard curve. A triplicate No-Template Control (NTC) using purified Milli-Q H_2O instead of a DNA sample was included on each plate.

CFX MaestroTM 3.1 (Bio-Rad) software was used to generate standard curves giving amplification efficiencies (E%) and R^2 values. Linear standard curves with $R^2 > 0.980$ and an E% between 90-110% (calculated as $E\% = 10^{[-1/slope]}$), and minute variation between replicates of the same concentration were considered acceptable for downstream analysis. Linearity (R^2) of the data provides a measure of variability between replicates of the same concentration and whether there is high amplification efficiency across replicates of different starting template copy numbers (Taylor *et al*., 2010). A perfect doubling of product during each qPCR cycle would yield an amplification efficiency of E% = 100% and $R^2 = 1$ (Svec *et al*., 2015). Efficiency of the telomere standard curve for the blood samples was 106.13%, $R^2 = 0.995$ generated from a mean of 3 standard curves with efficiencies of 101.7 – 110.5%, $R^2 = 0.993 – 0.996$. For the reference GAPDH gene the mean efficiency of the standard curves from blood samples was 99.67%, $R^2 = 0.995$ (E% = 97.2 – 102.7%, $R^2 = 0.994 – 0.997$). The mean telomere standard curve efficiency for sperm samples was E% = 103.5%, $R^2 = 0.997$ (E% = 100.9 – 107.8%, $R^2 = 0.996 – 0.997$). The mean GAPDH standard curve efficiency was E% = 108.2%, $R^2 = 0.994$ (E% = 106.5 – 110.7%, $R^2 = 0.992 – 0.996$).

Amplification of samples

On the day of qPCR amplification, DNA samples were diluted to 0.25ng/µl working stocks using purified Milli-Q H_2O. Each working stock was used for both telomere and GAPDH assays. Amplification of telomeres and GAPDH occurred on the same day, but on separate

plates as program details differed between them. DNA samples were run in triplicate on each plate. For corresponding telomere and GAPDH plates, the same samples were placed in the same well on both plates. All wells used had a total volume of 10µl, comprised of 4µl (1ng) of sample DNA and 6µl of a master mix including 5µl 1X SYBR® Green Supermix and 0.5µl of both forward and reverse primers per well (same concentrations as above). A master mix was prepared separately for each plate. A triplicate NTC and 3 separate Inter-Plate Calibrator (IPC) samples, run in triplicate, were included on each plate to be able to compare assays across all plates. IPC2 was used as a reference sample for calculating the T/S-ratio for all assays.

2.6 Analysing the data

2.6.1 Interpreting the results and calculating the T/S-ratio

CFX MaestroTM 3.1 (Bio-Rad) software was also used to collect and analyse data from the qPCR assays. Cq-values were obtained from each sample and the mean Cq calculated for each set of triplicates. If the standard deviation of the mean Cq-value surpassed the conventional 0.20, the sample was re-analysed on a later plate. If after three assays, the standard deviation of a sample remained high, it was removed from the overall analysis. However, three samples having standard deviations between 0.200 and 0.206 were included in the analysis due to the closeness of their variation to the accepted limit. The intra-plate coefficient of variation (CV%) between samples run in triplicate was 0.927% on average for telomeres (ranging from 0.038% to 1.860%, with n = 185 in total on all 10 plates, IPCs included) and 0.294% on average for GAPDH (0.011% – 0.743%, n = 185). Plate-to-plate consistency among the 10 assays was good, with an inter-plate CV% of 0.88% for IPC2 on average for telomeres (0.31% – 1.82%) and 0.39% on average for GAPDH (0.05% – 0.70%). The fluorescent signal for the NTCs did not exceed the threshold set by the software and revealed no inconsistencies.

The Pfaffl method (Pfaffl, 2001), was used to calculate the relative T/S-ratio for all samples and is described in the following equation from Cawthon (2002):

$$T/S - ratio = \frac{(E_{target})^{\Delta Cq_{target}}}{(E_{reference})^{\Delta Cq_{reference}}}$$

The amplification efficiency, as calculated from the standard curves by CFX Maestro™ 3.1, was used to generate a factor E = (E%/100%) + 1. ΔCq gives an estimate of how much each sample differs from the reference sample (IPC2 from each corresponding plate). It is calculated by subtracting the mean Cq-value of each sample from the mean Cq-value of the reference sample to obtain ΔCq_{target} (telomeres) and $\Delta Cq_{reference}$ (GAPDH) for each sample. All further statistical analyses use this relative T/S-ratio.

2.6.2 Statistical analyses

The inspection of data for normal distribution was done using a Shapiro-Wilks test and visual inspection of QQ-plots in SPSS (IBM, SPSS Statistics 25). All further statistical analyses and graphic illustrations were generated using R 3.4.4 (R Core Team, 2018). A significance level of 0.05 was used to reject the null hypothesis. The T/S ratio, hereafter termed relative telomere length (rTL), for blood samples was borderline normally distributed ($p = 0.053$), which improved after log transformation ($p = 0.80$). The rTL for sperm samples was not normally distributed ($p = 0.001$), even after log transformation ($p = 0.039$). However, upon inspection of the QQ-plot, the log transformation of rTL data was used in all analyses for ease of comparison with the results produced by Kristiane Hol.

All tests were performed for each year separately and combined. Relative telomere length of sperm was used as the response variable when analysing predictions related to blood, age, morphology, and rearing conditions (brood size). When examining reproductive success, fitness measurements such as clutch and brood size, mean nestling mass, number of fledglings, and laying date were used as response variables. Failed clutches or broods were excluded from such analyses, as the cause of loss was often difficult to determine and was not necessarily a result of parental quality. In addition, several nests in 2016 contained great tit chicks as part of a cross-fostering study and as such, these nests ($n = 6$) were excluded from all analyses pertaining to reproductive success.

Five individuals were caught in both 2016 and 2017 and were treated as separate data points in each year. Analysis of the change in rTL in these five individuals from one year to the next

was done using a Wilcoxon signed rank test. To control for pseudoreplication of the same individuals sampled in both years in combined analyses, individual identification (band number) was used as a random factor in linear mixed models (LMM). As the data was not always normally distributed, QQ-plots and homoscedasticity plots of LMMs were visually inspected to check that model assumptions were met. In cases where LMMs were not supported, one of the two data points from the two years was chosen at random, and non-parametric Spearmen correlations were performed. Testing the difference in rTLs between 2016 and 2017 was done using a Mann-Whitney U test (sperm) and a t-test (blood). Pearson and Spearman correlations were used for all other analyses, depending on whether the data was normally distributed or not.

3 Results

3.1 Sperm telomere length in relation to blood telomeres, year, and age

A comparison between sperm rTL and blood rTL from the same individuals revealed a significant positive correlation between the two (Spearman correlation, $r_s = 0.33$, $n = 40$, $p < 0.04$; Figure 1). Separating by year, the correlation was not significant in either year (2016: Spearman correlation, $r_s = 0.26$, $n = 23$, $p > 0.22$; 2017: Pearson correlation, $r = 0.24$, $n = 17$, $p > 0.35$). The sperm rTL was also not significantly different from blood rTL (Wilcoxon signed rank test, $V = 317$, $p > 0.80$). In 2017, sperm telomeres were on average 34% longer ($n = 24$) than in 2016 ($n = 26$) (Mann-Whitney U test, $U = 101$, $p < 0.0006$; Figure 2). Blood telomeres were 14% longer in 2017 ($n = 17$) than in 2016 ($n = 23$), but the difference was not statistically significant (t-test, $t = 1.65$, $p > 0.10$).

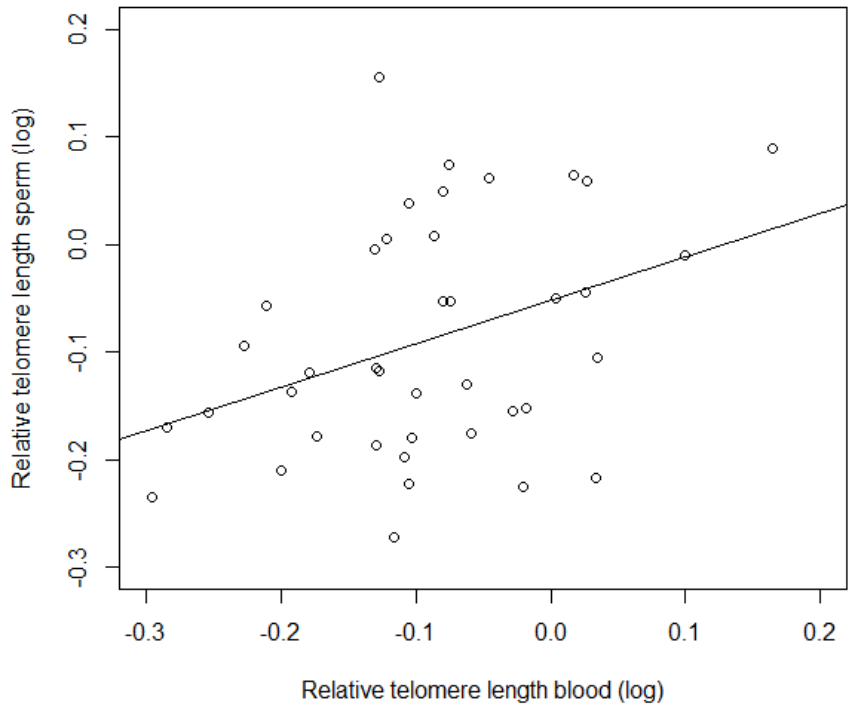

Figure 1. Relative telomere length in sperm in relation to relative telomere length in blood for 40 individual blue tits.

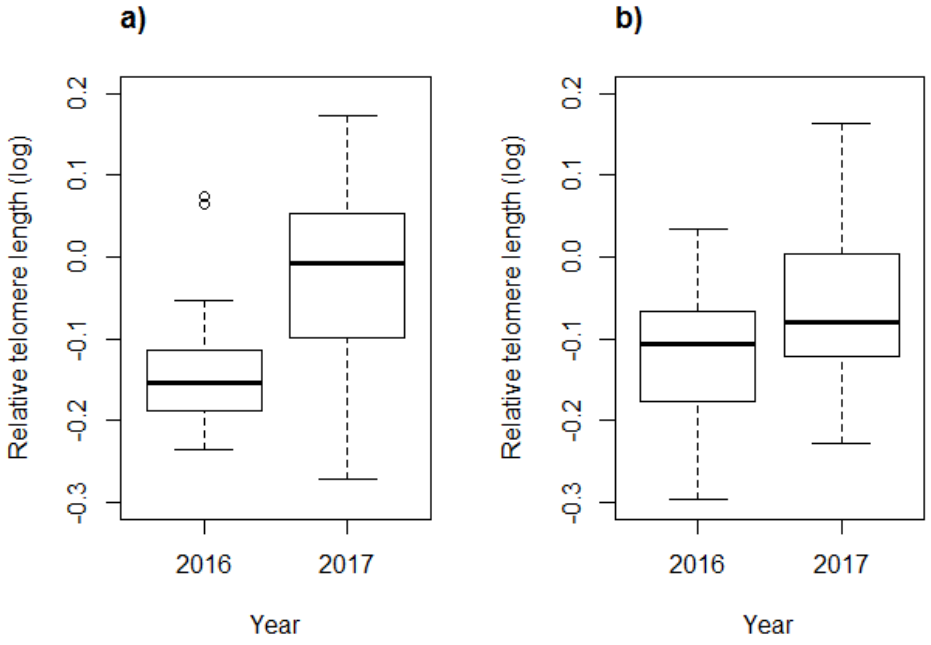

Figure 2. Boxplot showing the difference between years in relative telomere length for a) sperm (2016: n = 26; 2017: n = 24) and b) blood (2016: n = 23; 2017: n = 17) in blue tit males.

There was no association between rTL in sperm and age (Spearman correlation, $r_s = 0.01$, n = 45, p > 0.96; Figure 3), even when controlling for rTL in blood and year in a LMM (Table 2). This also did not change when analysing the two years separately (2016: Spearman correlation, $r_s = 0.17$, n = 26, p > 0.41; 2017: Pearson correlation, r = 0.07, n = 24, p > 0.74) nor when analysed as a polynomial regression in a LMM (Table 3).

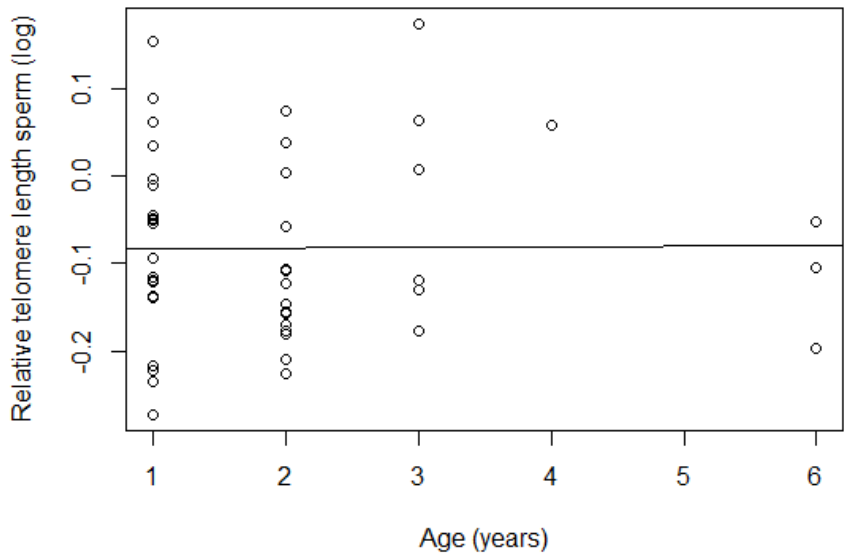

Figure 3. Relative telomere length in blue tit sperm in relation to the individual's age at time of sampling (n = 45).

Table 2. Linear mixed model of relative telomere length in blue tit sperm in relation to relative telomere length in blood, age, and year.

	Estimate	SE	df	t-value	Pr(>|t|)
(intercept)	-3.85	0.40	7.33	9.56	2.14e-05
rTL Blood	0.37	0.11	2.91	3.44	0.04
Age	0.03	0.02	34.86	1.34	0.19
Year	0.26	0.03	9.16	9.78	3.78e-06

Table 3. Linear mixed model for relative telomere length in blue tit sperm in relation to age and age2

	Estimate	SE	df	t-value	Pr(>|t|)
(intercept)	0.70	0.12	47	5.86	4.44e-07
Age	0.13	0.10	47	1.36	0.18
Age^2	-0.02	0.01	47	1.21	0.23

Longitudinal analysis of the five individuals caught both years yielded a marginally non-significant systematic increase in telomere length from 2016 to 2017 (Wilcoxon signed rank test, V = 0, n = 5, p = 0.063; Figure 4).

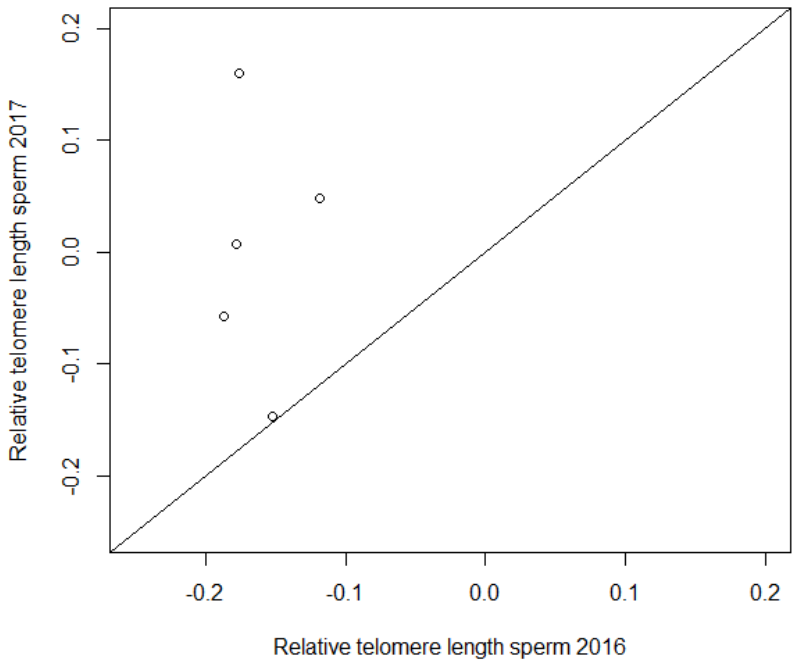

Figure 4. Relationship between the relative telomere length in sperm of male blue tits in 2016 caught again in 2017 (n = 5). Black line represents the line of unity.

3.2 Sperm telomere length in relation to rearing conditions and morphology

Sperm telomere length as an adult proved not to be correlated with rearing conditions (brood size as a chick) ($p = 0.15$; test statistics in Table 3). This held for 2016 ($p = 0.14$), but in 2017 there was a marginally non-significant trend ($p = 0.068$) between sperm telomeres and brood size as a chick, with those raised in larger broods having shorter sperm telomeres as adults (Figure 5; Table 3). Sperm telomere length was not related to body mass ($p = 0.16$), tarsus length ($p = 0.16$), or wing length ($p = 0.17$) as an adult (Table 3). This was also true when investigating each year separately (Table 3).

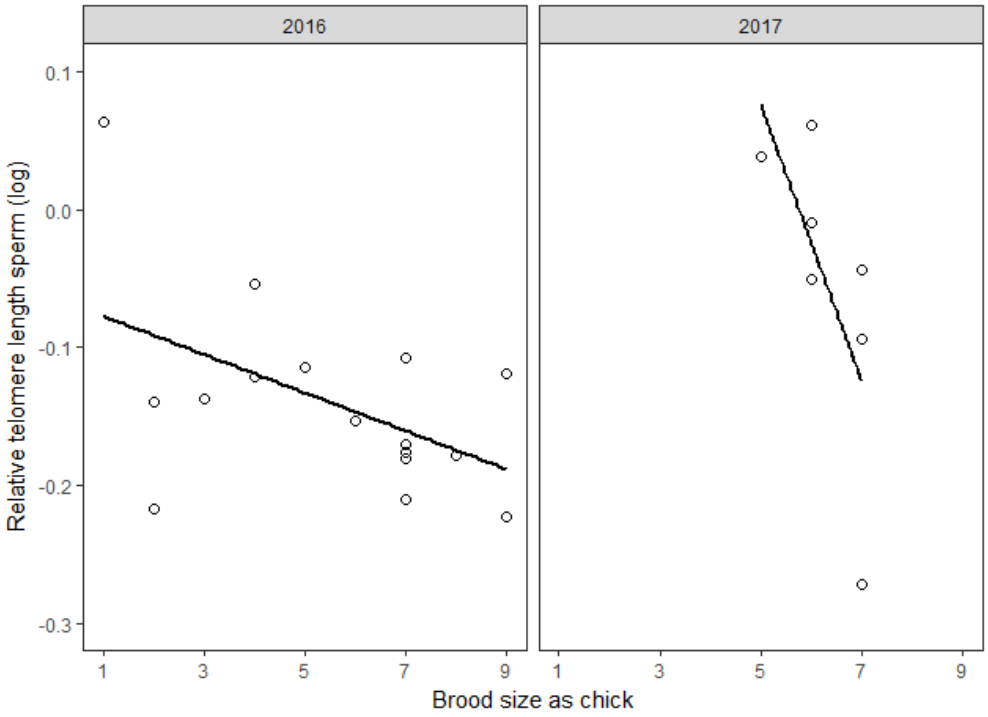

Figure 5. Relative telomere length in sperm of blue tits in relation to the brood size at day 15 of the individual as a chick; shown separately for the two years of study (2016: n = 16; 2017: n = 7).

Table 3. Summary of correlations between various traits and relative telomere length in sperm of blue tits. Spearman correlation (s) was used when one or both variables were not normally distributed, and Pearson correlation (p) was used when both variables were normally distributed.

Hypotheses	Variable	Year	n	r/r_s	p
Rearing conditions	Brood size as chick	Combined	21	-0.32^s	0.15
		2016	16	-0.39^s	0.14
		2017	7	-0.72^p	0.068
Morphology	Body mass (g)	Combined	45	-0.21^s	0.16
		2016	26	-0.26^s	0.21
		2017	24	-0.02^p	0.91
	Tarsus length (mm)	Combined	45	-0.21^s	0.16
		2016	26	0.08^s	0.68
		2017	24	-0.04^p	0.86
	Wing length (mm)	Combined	45	-0.21^s	0.17
		2016	26	-0.03^s	0.90
		2017	24	-0.09^p	0.69
Reproductive success	Laying date	Combined	38	0.14^s	0.40
		2016	21	-0.16^s	0.48
		2017	21	0.04^p	0.86
	Clutch size	Combined	38	0.09^s	0.61
		2016	21	0.41^s	0.08
		2017	21	0.01^s	0.98
	Brood size	Combined	36	0.11^s	0.51
		2016	20	0.06^s	0.80
		2017	20	0.37^s	0.11
	Number fledglings	Combined	31	-0.01^s	0.94
		2016	16	-0.33^s	0.21
		2017	18	0.26^p	0.30
	Mean nestling mass (g)	Combined	31	-0.24^s	0.18
		2016	16	0.31^s	0.24
		2017	18	-0.51^p	0.029

3.3 Sperm telomere length in relations to reproductive success

Sperm rTL was not correlated with laying date, clutch or brood size, or number of fledglings as a parent when analyzing the two years together nor when separate (Table 3). Sperm telomere length of an individual also did not predict the mean nestling mass of their brood at day 15 when examining both years combined ($p = 0.18$; Table 3) nor in 2016 ($p = 0.24$; Table 3). However, in 2017 males with longer telomeres had broods with significantly lower mean nestling masses ($p = 0.029$; Table 3; Figure 6).

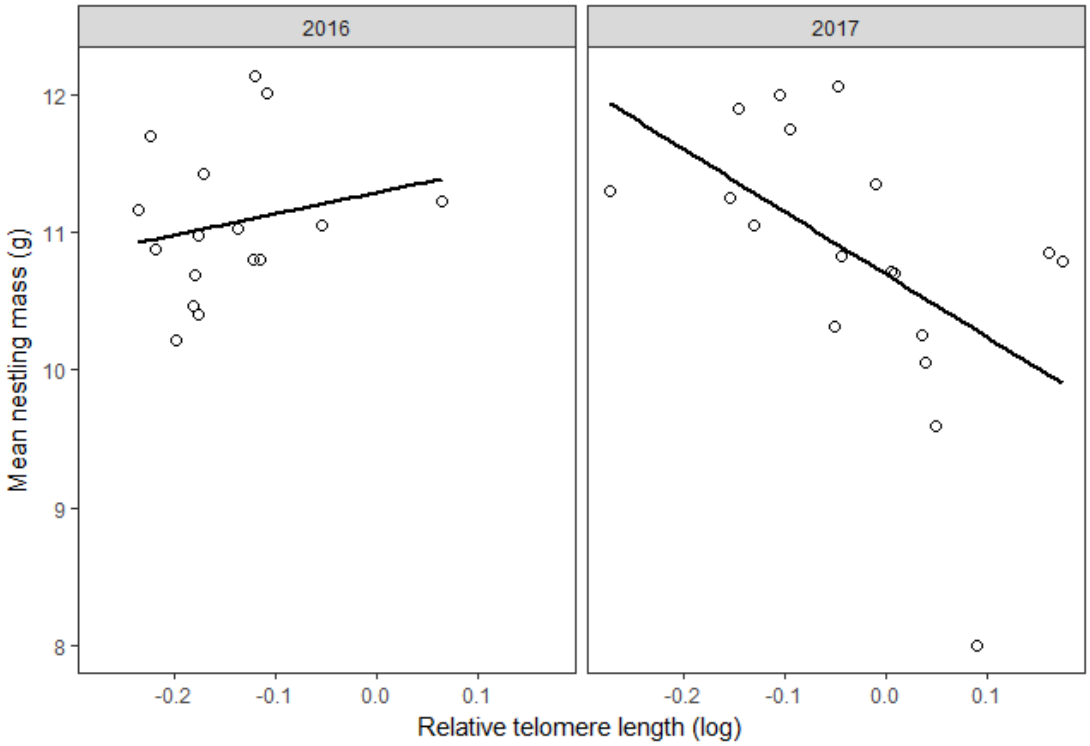

Figure 6. Mean nestling mass of each brood at day 15 in relation to the relative telomere length in blue tit sperm shown separately for the two years of study (2016: n = 15; 2017: n = 18).

4 Discussion

This study investigated telomere length in sperm of blue tits in relation to a number of biological factors. Sperm telomere length was found to be positively correlated with telomere length in blood cells, and not significantly different in length. Furthermore, individuals that were caught in 2017 were found to have longer sperm and blood telomeres than individuals caught in 2016. The age prediction was not supported as there was no relationship between relative telomere length in sperm and age in a cross-sectional analysis of the population. However, a longitudinal analysis of five individuals caught in both breeding seasons showed an increase in sperm telomere length from one year to the next. When assessing individual male attributes, sperm telomere length in blue tits was not significantly correlated with rearing conditions or morphology. Finally, mean nestling mass of the brood was the sole measurement of reproductive success that was correlated with telomere length in sperm and was only significant in one of the two study years.

The results showed that telomere length in blue tit sperm cells was positively correlated with telomere length in blood cells within an individual. This has also been demonstrated in two studies on humans where synchrony between leukocyte telomere length and sperm telomere length within individual men was found (Aston *et al*., 2012; Ferlin *et al*., 2013). Although the mean sperm telomere length was reported as being significantly longer than the mean leukocyte telomere length in the study on humans by Ferlin *et al*. (2013), variation among individuals was strong, with individuals with longer leukocyte telomeres generally having longer sperm telomeres. In the present study, the telomere length of sperm and blood cells were not significantly different from each other. Although one cannot directly compare the biology of long-lived humans and short-lived blue tits, one might speculate whether lifespan differences could explain the larger divergence in telomere length between sperm and blood in humans and other mammals (Kozik *et al*., 1998; Fradiani *et al*., 2004).

Relative telomere length in sperm was longer in 2017 than 2016. This was also true for the rTL in blood cells of the same individuals, although not statistically significant. However, Hol (2018) found a significant difference between 2016 and 2017 in a larger sample of male and female blue tit blood samples, hence the lack of significance in this study was most likely due to the smaller sample size. In a population of bluethroats (*Luscinia svecica*), a similar difference between years was also found in rTL of blood samples (Johnsen *et al*., 2017). The reasons for the annual differences are unknown, but they can hardly be explained by

differences in methods used, as samples from both years were processed at the same time, using the same method. The differences between the years may have been due to dissimilar levels of stress endured by the birds during the winters preceding their sampling. Johnsen *et al*. (2017) proposed that a year difference could be caused by a quality-difference in the individuals returning to the breeding site in both years of study. As sperm rTL was found to be correlated to blood rTL, one can speculate that the factors affecting telomeres in blood may also have the same effect on sperm telomeres.

Sperm rTL in blue tits showed considerable inter-individual variation at each age class, especially among first, second, and third year birds. However, no correlation between individual telomere length in sperm and age was found. Lack of data for the older cohorts (4+ years) may have had an influence on these trends (or absence thereof). Hol (2018) found similar results when comparing age and telomere length in blood cells of birds from the same population in a bigger sample including the blood samples used in the present study. These findings do not support what is previously known about telomere dynamics in sperm and blood in relation to age. In humans it has been well documented that there is a divergence in telomere length between somatic cells and germline cells after birth. Telomere length in somatic cells and hematopoietic (blood) stem cells is known to shorten with age, while sperm telomere length increases with age of men (Allsopp *et al*., 1992; Kimura *et al*., 2008; Aston *et al*., 2012; Ferlin *et al*., 2013), which has been attributed to the repression of the enzyme telomerase in somatic cells and the high activity of telomerase in the male germline. Aston *et al*. (2012) proposed that telomerase may only act to replenish the amount of telomeric repeats lost during replication and thus maintain telomeres at a relatively constant length. Short lived species have been shown to experience faster telomere shortening than long-lived species, with blue tits having one of the highest rates reported for birds (Haussmann *et al*., 2003; Sudyka *et al*., 2016; Tricola *et al*., 2018). With such high telomere erosion rates, telomerase may only have the capacity to replenish what has been lost and may explain the lack of correlation between sperm rTL and age in blue tits in the present study. One can also not rule out the possibility that a larger sample size and equal representation across all age classes would have revealed a pattern similar to that found in humans.

While the above cross-sectional analysis of sperm rTL and age revealed no correlation, a longitudinal inspection of five males that were caught in both field seasons showed an increase in sperm rTL for all individuals from 2016 to 2017. This trend was borderline non-

significant and with such a small sample size it is difficult to assess validity of the results. Even so, one cannot ignore that an increase in rTL was seen for all five individuals in the analysis and perhaps a larger sample size would have unveiled a significant increase. The role of telomerase, as hypothesized, may be to replenish those telomeric repeats lost during cell replication, but this does not explain the elongation of sperm telomeres that is seen from one year to the next, and as such there must be an additional effort of telomerase. In addition to telomere maintenance, an 'over-activation' of telomerase in the male germ cells has been proposed, which results in the elongation of telomeres with the replication of sperm precursors (Aston *et al*., 2012). Perhaps an over-activation of telomerase occurs for strong, healthy individuals that experience less stress-related attrition and thus over time undergo telomere lengthening in their sperm.

As a male becomes older, telomere lengthening may reflect a stable environment and therefore a male should provide their offspring with longer telomeres when the prospects for a long life are better. However, in order for this life history hypothesis to be valid it should be costly for males to produce sperm with long telomeres, otherwise all individuals would do so. Studies on humans have revealed the effects of paternal age and telomere length on offspring telomere lengths, where older men tend to have offspring with longer telomeres (Unryn *et al*., 2005; De Meyer *et al*., 2007; Kimura *et al*., 2008; Nordfjäll *et al*., 2010). Although there was no correlation between sperm rTL and age in blue tits within the present population, increasing telomere length in sperm within an individual as they age may have a potential effect on offspring by passing on longer telomeres which may act to reduce age-related disorders and increase longevity in offspring of older fathers (Kimura *et al*., 2008; Sartorius & Nieschlag, 2010). In addition, as there is large variation in sperm rTL and no relationship with age within the populations, but individual elongation from one year to the next, there is an indication that sperm telomere length is more of a reflection of biological age rather than chronological age (Bize *et al*., 2009, Bauch *et al*., 2013).

A marginally non-significant correlation ($p = 0.068$) was found between sperm rTL in 2017 and brood size experienced as a chick, suggesting that males raised in larger broods have shorter sperm telomeres as adults than those from smaller broods. This correlation was also not significant for 2016 or in the combined data set; however, there was a similar negative trend in the data. In support, for a larger sample of blue tits, Hol (2018) also found that males that were reared in larger broods had shorter blood rTL as adults ($n = 29$), but this did not

hold for the small sample of females (n = 9). The negative effect of brood size on telomere length in nestlings has been seen in other avian species (Boonekamp *et al*., 2014; Watson *et al*., 2015; Young *et al*., 2017). Telomere loss has been shown to be more rapid during the early phase of life, which has been attributed to rapid cell division during the growth period (Zeichner *et al*., 1999; Salomons *et al*., 2009; Johnsen *et al*., 2017) and oxidative stress resulting from increased brood sizes can accelerate telomere attrition (Boonekamp *et al*., 2014; Hall *et al*., 2004). Although the sample size (n = 7) and variation in brood size (5 – 7 nestlings) were both small in 2017, analysis of the combined data from both years covered a broader spectrum of brood sizes and was still not significant. A larger sample would shed light on whether this pattern is biologically significantly or not.

Relative telomere length in sperm was not correlated with any of the morphological measures and, to the best my knowledge, there is no previous study on such relationships. Body morphology may be seen as a measurement of health and quality and as such one might hypothesize that this may be reflected in telomere length in somatic cells, and thus sperm cells as they have been found to be correlated in humans (Aston *et al*., 2012; Ferlin *et al*., 2013) and birds (present study). To compare with analyses of rTL in blood of the blue tits, Hol (2018) only found a significant correlation with wing length in males, and most notably in one-year-old birds. Aabye (2017) found no such relationship when performing a preliminary study on a smaller sample of the same population. A study on bluethroats by Johnsen *et al*. (2017) also investigated telomere length in blood in relation to morphological traits and found no such significant correlations.

Even though laying date and clutch size are ultimately more influenced by characteristics of the female than the male, she may adjust such parameters based on the quality and attractiveness of her mate (Burley, 1986; Horváthová *et al*., 2012). Attractiveness may be an indication of genetic quality which could be reflected in telomere length in sperm, so from the differential allocation hypothesis (Burley, 1986), one would predict that females would lay larger clutches when mated with a male with longer sperm rTL. Although attractiveness was not tested in this study, rTL in sperm did not seem to have an effect on laying date or the size of clutch laid by the female; unlike what was seen by Hol (2018) who found that female blue tits that produced larger clutches were mated with males that had longer blood rTL. One problem is how a female would be able to assess such male qualities. Possible candidates to be investigated, in addition to the above mentioned morphological traits and age, are male

plumage characters, behavior, and territory quality. When testing other measures of reproductive success, no relationship was found between sperm rTL and brood size or number of fledglings as an adult in this population.

Blue tit males with longer sperm rTL were found to have raised broods with a lower mean nestling mass in the 2017 breeding season. This result is hard to explain if telomere length reflects high individual quality, and/or high quality of their territories and mates. One may speculate that in this blue tit population, males with longer sperm rTL may have mated with older or lower quality females, resulting in inferior parental care and lower mean nestling masses. Older blue tit females (4+ years) have been shown to have lower nestling success and decreased post-fledging survival (Dhondt, 1989) and in a study by Slagsvold and Lifjeld (1990), great tit females who were handicapped (some feathers removed) fledged young with significantly reduced masses. Criscuolo *et al*. (2017) found that Alpine swift (*Apus melba*) nestlings' telomere length was negatively associated with the age of the mother that reared them. They suggested that older females may have provided suboptimal parental care to their offspring resulting in increased telomere erosion. However, upon analysis there was no correlation between sperm telomere length in male blue tits and the age of their mate (Spearman correlation, $r_s = -0.05$, $n = 18$, $p > 0.83$). Furthermore, the relationship between sperm rTL and mean nestling mass in 2017 was one of the few significant results obtained among the many test performed in the present study. When accounting for the problem of multiple comparisons per variable (two years and combined), using Bonferroni correction, the critical significance level was 0.017, making this result ($p = 0.029$) marginally non-significant. Obviously, a larger sample and more study years are needed to test if this result is biologically meaningful, which seems unlikely given the inconsistencies between the two breeding seasons (positive but non-significant trend in 2016, negative trend in 2017).

5 Conclusion

This study is the first to investigate sperm telomere relationships in birds. Although preliminary, it shows that there is a positive correlation between telomeres length in blood and sperm cells across individuals, and that undefined factors can have an influence on telomere length between seasons. It also suggests that sperm telomere length and elongation is more representative of biological age rather than chronological age, as there was no relationship between sperm telomere length and age across the population, while elongation was seen on an individual level in five males between the two study seasons. A larger data set and a more equal representation across the age classes would help to obtain a stronger test of these trends. More data is also needed to disentangle the inconsistencies between seasons on other factors such as rearing conditions and reproductive success. Future studies should also investigate the influence of sperm telomeres on offspring telomere length and a potential generational effect.

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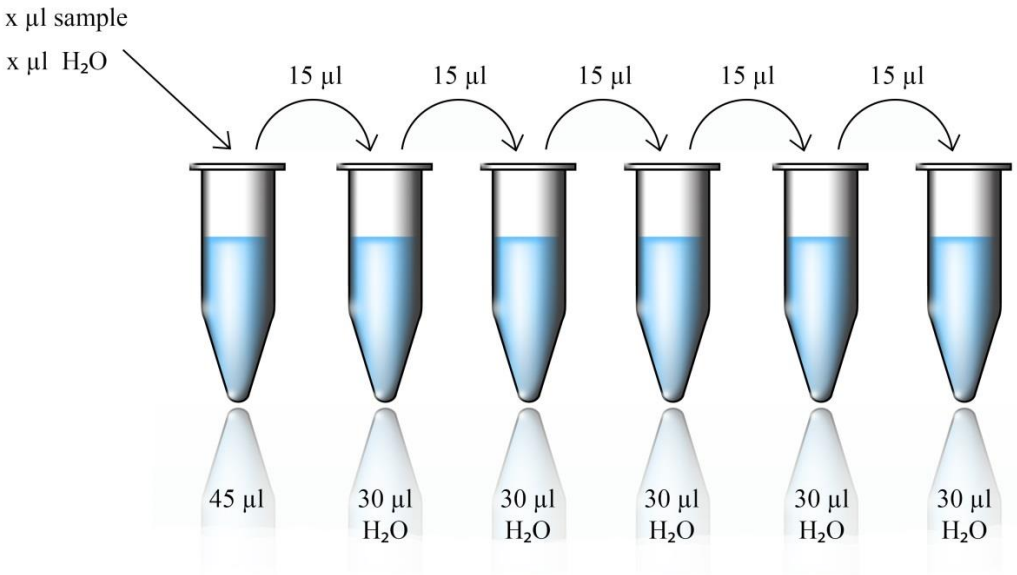
Appendix

1) Serial dilution for standard curves

One DNA sample was used to create separate 45µl stocks of 1 ng/ µl (telomeres) and 2 ng/ µl (GAPDH), which were used as the basis for serial dilution (1:3) with six replicates and six duplicates at each concentration. The volume of sample and H_2O added to the first tube is determined by the concentration of the DNA sample so there is a final volume of 45 µl and a concentration of either 1 ng/ µl or 2 ng/ µl. Separate standard curves were made for sperm and blood. Diagram used with permission from Kristiane Hol (2018).



2) Output from CFX^{TM} Maestro

All samples included in the study and their corresponding qPCR plate on which they were run. The samples were processed in one of ten sessions and those with a standard deviation above 0.2 (rule of thumb) were re-processed on plate eight, nine, or ten. Samples with a standard deviation above 0.2 upon second evaluation were excluded from the data set, with the exception of three samples having standard deviation between 0.200 and 0.206 (not

shown). Each run included two plates: one for amplification with telomere-primers and one with GAPDH-primers. All samples and IPCs were processed in triplicate on each plate and the mean Cq was calculated by the program. Sample 91451 was used as the IPC and is marked in yellow.

Plate 1

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.20	0.032	26.58	0.028
Blood	91459	10.81	0.028	27.23	0.05
Blood	91456	11.45	0.172	27.13	0.061
Blood	91479	11.21	0.132	27.25	0.041
Blood	91445	10.45	0.052	26.48	0.083
Blood	91482	10.37	0.153	27.09	0.075
Blood	91461	10.51	0.155	26.99	0.095
Blood	91449	9.83	0.031	26.74	0.065

Plate 2

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.83	0.144	27.74	0.155
Blood	86101	11.27	0.081	27.77	0.071
Blood	91465	11.15	0.158	27.37	0.012
Blood	86106	10.88	0.082	27.37	0.069
Blood	91474	10.50	0.080	27.13	0.018
Blood	91481	10.83	0.034	27.39	0.006
Blood	91458	10.54	0.186	27.23	0.110
Blood	91463	10.29	0.033	27.08	0.088

Plate 4

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	12.18	0.042	27.85	0.051
Blood	86050	11.02	0.133	26.75	0.169
Blood	86051	11.28	0.156	26.84	0.083

Plate 5

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.93	0.109	27.25	0.013
Blood	86059	11.26	0.050	26.75	0.090
Blood	86061	10.63	0.072	26.67	0.036
Blood	86063	10.57	0.086	26.54	0.042
Blood	86064	10.85	0.047	26.57	0.055
Blood	86067	11.06	0.157	26.40	0.075
Blood	86070	10.32	0.010	26.27	0.145
Blood	86073	10.91	0.186	26.28	0.060
Blood	86080	11.10	0.027	26.76	0.039
Blood	86090	10.98	0.110	26.95	0.062
Blood	86091	11.13	0.084	27.21	0.014
Blood	86098	10.73	0.076	26.61	0.109
Blood	86099	10.34	0.030	26.69	0.052
Blood	86103	10.74	0.073	26.80	0.157

Plate 6

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.33	0.090	27.14	0.090
Sperm	91447	11.05	0.083	27.45	0.172
Sperm	91481	10.65	0.079	27.57	0.060
Sperm	86078	10.64	0.131	26.88	0.003
Sperm	91480	10.73	0.105	27.55	0.093
Sperm	86086	10.97	0.066	27.28	0.093
Sperm	91475	10.95	0.122	27.28	0.105
Sperm	86089	10.86	0.202	27.10	0.065
Sperm	91478	10.39	0.004	27.70	0.115
Sperm	86101	11.04	0.064	27.24	0.065
Sperm	91465	10.61	0.091	27.23	0.051
Sperm	86106	10.60	0.069	27.03	0.026
Sperm	91474	10.72	0.012	27.67	0.016
Sperm	86050	10.85	0.122	26.96	0.098
Sperm	86051	12.26	0.165	28.30	0.062
Sperm	86054	10.88	0.082	27.29	0.034
Sperm	86059	10.93	0.077	27.23	0.119
Sperm	86061	10.49	0.013	27.13	0.096
Sperm	86063	10.75	0.147	27.11	0.088

Plate 7

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.46	0.134	27.25	0.190
Sperm	86064	10.64	0.056	27.05	0.040
Sperm	86067	11.71	0.093	27.72	0.071
Sperm	86069	10.44	0.027	26.61	0.077
Sperm	86070	10.59	0.060	26.81	0.083
Sperm	86071	10.78	0.061	27.13	0.017
Sperm	86073	11.60	0.074	27.82	0.056
Sperm	86080	11.07	0.143	27.18	0.064
Sperm	86084	10.69	0.135	27.14	0.015
Sperm	86091	10.53	0.161	27.15	0.056
Sperm	86093	10.55	0.014	27.00	0.103
Sperm	86098	10.76	0.107	27.18	0.037
Sperm	86099	10.48	0.095	27.47	0.092
Sperm	86102	10.99	0.057	27.21	0.125
Sperm	86103	10.59	0.070	27.61	0.064
Sperm	91445	10.24	0.069	27.23	0.086
Sperm	91449	10.21	0.198	27.29	0.137
Sperm	91450	11.30	0.098	28.05	0.122
Sperm	91451	10.64	0.118	27.97	0.118
Sperm	91453	10.03	0.142	27.32	0.115
Sperm	91455	10.44	0.060	27.34	0.123

Plate 8

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	11.02	0.201	27.55	0.178
Sperm	91456	11.13	0.133	27.36	0.095
Sperm	91458	10.74	0.074	26.87	0.051
Sperm	91459	11.04	0.056	27.41	0.044
Sperm	91460	10.23	0.166	26.80	0.077
Sperm	91461	11.38	0.012	27.76	0.124
Sperm	91463	11.77	0.188	27.79	0.056
Sperm	91464	10.80	0.058	27.52	0.150
Sperm	91471	10.99	0.149	27.37	0.090
Sperm	86090	11.03	0.105	26.86	0.034
Blood	91450	11.34	0.096	27.45	0.108
Blood	86086	11.07	0.063	27.54	0.037
Blood	86089	11.16	0.147	27.50	0.178

Plate 9

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.72	0.082	27.42	0.111
Blood	86078	11.53	0.090	27.69	0.094
Blood	91464	12.23	0.181	29.09	0.020
Blood	86069	11.18	0.006	27.54	0.095
Blood	86071	11.75	0.153	27.67	0.033
Blood	86084	10.86	0.037	27.68	0.051

Plate 10

	Sample	Telomere Mean Cq	Telomere St. dev.	GAPDH Mean Cq	GAPDH St. dev.
	91451	10.89	0.044	27.34	0.100
Sperm	91462	10.94	0.086	27.06	0.048
Sperm	91479	11.05	0.126	26.64	0.042
Sperm	91482	10.25	0.139	26.69	0.037
Blood	91453	11.42	0.162	27.47	0.061
Blood	91480	10.84	0.172	27.00	0.077
Blood	91460	11.24	0.117	27.30	0.063

3) Complete data set

Sample	Collection date	Age at sampling (minimum)	Relative telomere length (Sperm)	Relative telomere length (Blood)	Plate (Sperm PCR amplification)	Plate (Blood PCR amplification)	Brood size (as chick)	Body mass (g)	Tarsus length (mm)	Wing length (mm)	Laying date (1 = 1 April)	Clutch size	Brood sized	Number of fledglings	Mean nestling mass (g) 15 days
86050	09.05.16	1	0.605957	1.08158	6	4	2	11.2	18.7	66	25	10	10	10	10.9
86051	09.05.16	2	0.595295	0.9537	6	4	-	11.9	19.7	71	-	-	-	-	-
86054	10.05.16	1	0.755608	-	6	-	4	10.1	19	68	19	11	4	4	10.8
86059	11.05.16	2	0.69788	0.55741	6	5	-	11.9	20.2	70	20	9	-	-	-
86061	11.05.16	6	0.886162	0.83188	6	5	-	11.8	20.2	71	-	-	-	-	-
86063	11.05.16	1	0.726153	0.79409	6	5	2	10.8	18.9	68	20	11	10	-	-
86064	11.05.16	1	0.760045	0.66209	7	5	9	11.9	20	70.5	24	11	10	5	12.1
86067	11.05.16	1	0.581452	0.5057	7	5	-	10.5	18.1	68	29	8	7	3	11.2
86069	11.05.16	6	0.634359	0.77899	7	5	-	11.8	19.6	71	33	9	9	9	10.2
86070	12.05.16	2	0.660405	0.78944	7	5	7	11.4	19.2	71	31	11	11	6	10.5
86071	12.05.16	1	0.729757	0.64277	7	5	3	11	19.1	71	36	7	7	7	11.0
86073	12.05.16	2	0.676484	0.51878	7	5	7	11	19.4	71	36	9	9	9	11.4
86078	13.05.16	2	0.663243	0.67086	6	2	8	10	18.9	69	19	10	9	9	10.7
86080	13.05.16	2	0.61623	0.63015	7	5	7	11.7	20.2	69	22	8	8	-	-
86084	13.05.16	2	0.783602	1.08169	7	5	-	11	20.9	70.5	26	10	10	-	-
86086	18.05.16	1	0.703687	0.95783	6	2	6	11.2	18.8	67	22	10	9	-	-
86089	18.05.16	2	0.666727	0.87298	6	2	7	11.2	19	68	19	10	8	7	10.4
86090	18.05.16	1	0.598642	0.78379	8	5	9	11.8	18.9	68	22	10	7	7	11.7
86091	18.05.16	1	0.884265	0.8417	7	5	4	9.8	18.4	67.5	22	11	10	4	11.1
86093	18.05.16	2	0.780994	-	7	-	7	11.7	18.9	69	24	8	8	8	12.0
86098	24.05.16	1	0.76783	0.74238	7	5	5	9.9	18.8	67	18	11	9	7	10.8
86099	24.05.16	3	1.158519	1.04032	7	5	1	11.3	19.4	70	25	11	3	3	11.2
86101	27.05.16	1	0.650235	0.74266	6	2	-	10.6	17.9	66	-	-	-	-	-
86102	27.05.16	3	0.666727	-	7	-	-	10.8	18.2	72	26	9	8	8	11.0
86103	27.05.16	2	1.187395	0.84052	7	5	-	10.2	18.8	67	-	-	-	-	-
86106	27.05.16	3	0.761676	0.74675	6	2		10.2	17.3	68.5	-	-	-	-	-
91445	05.05.17	1	1.151915	0.90004	7	1	6	10.80	18.6	67.5	29	10	-	-	-
91447	09.05.17	2	0.75313	-	6	-	-	10.80	19.9	67.5	32	12	11	-	-
91449	09.05.17	1	1.229628	1.45976	7	1	-	10.20	17.4	65	30	12	11	1	8.0
91450	09.05.17	1	0.990716	0.74036	7	1	-	10.30	17.9	68	-	-	-	-	-
91451	09.05.17	3	1.492249	-	7	-	-	10.20	18.1	69	37	8	7	7	10.8

Sample	Collection date	Age at sampling (minimum)	Relative telomere length (Sperm)	Relative telomere length (Blood)	Plate (Sperm PCR amplification)	Plate (Blood PCR amplification)	Brood size (as chick)	Body mass (g)	Tarsus length (mm)	Wing length (mm)	Laying date (1 = 1 April)	Clutch size	Brood sized	Number of fledglings	Mean nestling mass (g) 15 days
91453	09.05.17	1	1.428212	0.74567	7	1	-	11.90	20.6	68.5	-	-	-	-	-
91455	12.05.17	1	1.083493	-	7	-	-	11.50	20.1	70.5	30	11	10	9	10.3
91456	12.05.17	1	0.804625	0.59224	8	1	7	11.50	18.6	68	25	10	7	7	11.8
91458	12.05.17	3	0.740818	0.86685	8	2	-	10.70	17.1	70	31	10	10	10	11.1
91459	12.05.17	1	0.889713	1.00827	8	1	6	11.00	18.8	68	33	8	8	7	10.3
91460	19.05.17	2	1.010574	0.75516	8	2	-	11.50	18.8	68.5	25	8	8	8	10.7
91461	19.05.17	1	0.903549	1.06107	8	1	7	10.80	18.6	68	21	11	10	6	10.8
91462	19.05.17	6	0.785993	-	10	-	-	11.50	19	71	24	8	1	1	12.0
91463	19.05.17	2	0.700378	0.93633	8	2	-	11.30	19.1	70	31	11	2	1	11.3
91464	19.05.17	4	1.143499	1.06456	8	2	-	11.70	19.6	71	-	-	-	-	-
91465	25.05.17	2	0.875759	0.61427	6	2	-	10.60	18.3	69	31	8	8	-	-
91471	25.05.17	1	0.895195	-	8	-	-	10.80	17.5	68	32	8	8	8	12.1
91474	26.05.17	4	1.118462	0.83268	6	2	-	10.30	16.9	69	23	11	10	8	9.6
91475	26.05.17	2	0.713743	-	6	-	-	11.20	19.2	69	30	10	2	2	11.9
91478	26.05.17	3	1.444977	-	6	-	-	11.10	18.6	69	18	11	7	7	10.9
91479	26.05.17	1	0.534271	0.76547	10	1	7	10.70	19.6	68	20	12	6	5	11.3
91480	26.05.17	3	1.017003	0.8196	6	2	-	10.20	18	68	17	8	5	5	10.7
91481	26.05.17	2	1.092302	0.78504	6	2	5	11.40	19.2	69	31	12	11	11	10.1
91482	26.05.17	1	0.977677	1.25822	10	1	6	9.90	18.4	67	27	12	9	8	11.4