The silver spoon effect and individual quality in relation to telomere length in blue tits (*Cyanistes caeruleus*)

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Photo: Kristiane E. Hol

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- KRISTIANE E. HOL

Abstract

Telomeres shorten over time due to internal and external factors. They are linked to factors experienced by an organism throughout its lifetime and may explain variations among individuals in a number of life-history traits. This thesis aims to investigate the silver spoon effect and individual quality in adult blue tits (*Cyanistes caeruleus*) in relation to relative telomere length. In addition, I analysed the relationship between relative telomere length, morphology and survival, by using cross-sectional and longitudinal data. Field work was conducted at Dæli, near Oslo. Blood samples from 109 individuals collected in 2016 and 2017 were assessed using a qPCR assay to estimate relative telomere length, and correlations between relative telomere length and various variables were performed. There were significant year and collection date effects on telomere length. Furthermore, the results indicate that there is a silver spoon effect on telomere length, because blue tits growing up in larger broods had shorter telomeres as adults than those growing up in smaller broods. In terms of individual quality, adult males with longer telomeres mated with females that laid larger clutches; however, one-year-old males with longer wings had shorter telomeres. I discuss possible explanations for these somewhat inconsistent results.

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1 Introduction

Conditions experienced early in life, when the individual is under development, have consequences for life history traits, such as survival and later reproduction (Lindström, 1999). Good conditions experienced early in life have been referred to as the silver spoon effect, which is defined as the life-long reproductive advantage (i.e. increased fitness) enjoyed by an individual who had access to abundant resources during the developmental stage of its life (Monaghan, 2008). Early life conditions can vary as a result of parent quality (Van De Pol *et al.*, 2006), territory quality, and food availability (Svensson & Nilsson, 1995; Young *et al.*, 2017). Traits expressed by parents often form a large part of the offspring's environment. There is a trade-off of different life history traits and the parents should adjust their current reproduction according to the expected pay-off and expected future reproduction (Stearns, 1992). Different strategies are usually divided between the optimal resource use on growth, reproduction, and self-maintenance, and there is also a trade-off between number and size of offspring (Stearns, 1992). Organismal processes are linked to telomeres, repetitive DNA-sequences at the end of chromosomes (Blackburn, 1991), which might explain variation in a number of life-history traits. Telomeres are lost through cell division (Watson, 1972) and oxidative stress (Bar-Or *et al.*, 2001; von Zglinicki, 2002), and are related to cellular aging (Aubert & Lansdorp, 2008), survival (Bize *et al.*, 2009; Salomons *et al.*, 2009), reproductive success (Pauliny *et al.*, 2006), and growth (Hall *et al.*, 2004).

Telomeres are repeated nucleotide sequences with a G-rich strand located at the end of eukaryotic chromosomes. They are thousands of base pairs long and composed of tandem repeats of $(AGGGTT)_n$ sequences in vertebrates; the number repeats determines the length of the telomere (Blackburn, 1991). Telomeres shorten with each cell division due to the inability of DNA polymerase to completely replicate the end of the new lagging strand, referred to as the end replication problem (Watson, 1972). The role of telomeres is to prevent chromosome degradation and fusion (Blackburn, 1991). Eventually, the telomeres shorten to a point where cellular senescence occurs (Hornsby, 2002) (see Appendix 1 for more details on telomeres). However, cell division is not the only cause of telomere shortening. Stress factors like oxidative stress, UV-radiation, and alkylation can induce telomeric double-stranded breaks (Bar-Or *et al.*, 2001; von Zglinicki, 2002). The rate of telomere shortening per cell division is not constant, but changes from cell to cell, and most likely between division cycles. This is because of (external) oxidative stress and (internal) antioxidative defences. Oxidative damage contributes, in many cases, more to telomere loss than the end-replication problem. This type

of telomere shortening will always be accompanied by significant damage elsewhere in the genome (von Zglinicki, 2002). The amount of unrepaired oxidative damage to the telomeres influence the loss at the next cell division (von Zglinicki, 2002). Oxidative stress has been shown experimentally to increase telomere loss *in vivo* in mice (*Mus musculus castaneus*) (Cattan *et al.*, 2008), as well as *in vitro* in fibroblasts of sheep (*Ovis aries*) and humans (Richter & von Zglinicki, 2007).

Telomere lengths can be restored through the enzyme telomerase, a ribonucleoprotein capable of rebuilding telomeres by adding TTAGGG repeats (Greider & Blackburn, 1985). However, telomerase activity is not high enough to prevent telomere loss in most cell lines, including blood cells. Telomeres in blood cells thus shorten with age (Lansdorp, 2005). Even though telomerase activity seems to be essential for telomere maintenance, it is repressed in most somatic tissue, probably as a mechanism to prevent tumor growth (Kim *et al.*, 1994). In birds, blood samples are frequently used for telomere studies due to the fact that avian erythrocytes (red blood cells) contain nuclear DNA and only a small amount of blood is required. In zebra finches (*Taeniopygia guttata*) it has been found that the telomere length in red blood cells is related to telomere length in other somatic tissues (Reichert *et al.*, 2013).

Similar to other vertebrates, telomeres shorten throughout the course of life in birds (Haussmann *et al.*, 2003; Sudyka *et al.*, 2016). However, there are large variations in telomere length within the same age groups, which indicate that telomere length reflects biological age rather than chronological age (Bize *et al.*, 2009; Bauch *et al.*, 2013). A study on the lesser black-backed gull (*Larus fuscus*) showed that males tend to have longer telomeres than females as nestlings (Foote *et al.*, 2011). A connection between early life telomere length and longevity was found in zebra finches (Heidinger *et al.*, 2012), and a longitudinal study on jackdaws *(Corvus monedula*) (Salomons *et al.*, 2009) showed that telomere loss is more rapid early in life, probably due to the high rate of cell division during the growth period. Long telomeres in fledglings can therefore be a good predictor of lifespan (Heidinger *et al.*, 2012; Young *et al.*, 2017).

Good environmental conditions growing up can be important for further survival. It has been found that nestlings get shorter telomeres when reared under unfavourable conditions (Boonekamp *et al.*, 2014; Watson *et al.*, 2015; Salmón *et al.*, 2016). Rearing conditions can also have long term consequences on adult morphology because the bone structure and tarsus lengths are fully developed in passerine birds before fledging (Garnett, 1981). Tail and wing

length have also been shown to be positively related to relative telomere length in barn swallows (*Hirundo rustica*) (Parolini *et al.*, 2015). High body mass in adults may indicate good individual condition and fat deposition, which is often associated with high fitness (Moya-Laraño *et al.*, 2008). Reproduction has been shown to lead to loss of telomeres (Heidinger *et al.*, 2012; Bauch *et al.*, 2013; Sudyka *et al.*, 2014), but individual quality and factors such as stress and disease also affect telomere shortening (Bauch *et al.*, 2013; Nettle *et al.*, 2013; Asghar *et al.*, 2015).

Blue tits (*Cyanistes caeruleus*) lay primarily one clutch per year (Haftorn, 1971), so time of breeding is an important fitness factor. Earlier hatching in blue tits is associated with higher fledgling survival and recruitment in the following year (Svensson & Nilsson, 1995). Blue tit nestling body condition may influence short term survival and predict long term weight and fat deposition (Merilä & Svensson, 1997). Blue tits given nutritional supplements produced higher fledgling success that season and experienced reduced telomere erosion rates in the following year compared to a control group, and also compared to another experimental group given antimalaria medication (Badás *et al.*, 2015). In another study of blue tits, parents with experimentally increased brood sizes had significantly shorter telomeres after rearing compared to the control group (Sudyka *et al.*, 2014). The shortening of telomeres can therefore reflect a cost of reproduction in blue tits.

This study investigates blue tit telomere length dynamics in relation to age, sex, year, and collection date. I test two hypotheses for explaining telomere length variation: the silver spoon effect and telomeres as a quality indicator. The silver spoon effect focuses on rearing conditions. I predict that if there is a silver spoon effect on adult telomere length, telomere length will be positively related to the rearing conditions experienced, estimated by hatching date, number of siblings and average body mass as nestlings. If telomeres indicate individual quality, I predict that telomere length in parents is associated with early laying and hatching date, and clutch and brood size. In addition, I will test two sets of predictions that fit both hypotheses. First, I will test if telomere length is positively associated with morphology and quality, estimated by wing length, tarsus length and body mass. Second, I will investigate if individuals with longer telomeres have a higher survival rate from one breeding season to the next.

2 Materials and Methods

2.1 Study species and study area

Blue tits are small cavity-nesting passerines. Adults weigh 10-13 g, with males being slightly larger and more colourful than females (Haftorn, 1971; Andersson *et al.*, 1998). In spring, the female builds the nest and lays 4-14 eggs and incubates them for 12-16 days. The male feeds the female both before egg laying and during incubation. Once hatched, the nestlings are fed by both parents before they leave the nest after 16-22 days (Haftorn, 1971).

For this study, field work was conducted between the end of March until the end of June in 2017 at Dæli (59°56'N, 10°32'E), near Oslo. The study area is about 1.6 km^2 and is part of a woodland area dominated by deciduous trees with some patches of coniferous trees. The area contains approximately 550 wooden nest boxes attached to tree trunks about 1.5 m above ground, and positioned 40-50 m apart throughout the area (Slagsvold *et al.*, 2013).

The monitoring of the local blue tit population at Dæli began in 1995 and continues to this day. Blue tits in this population have a survival rate of about 46 % and approximately 5-10 % of local fledglings return to the area as local recruits the following year. Although only blue tis were assessed in this study, other hole-nesting passerines were nesting in the provided nest boxes. In the spring of 2017, 120 blue tits and 64 great tits (*Parus major*) were nesting in the provided boxes, as well as 49 pied flycatchers (*Ficedula hypoleuca*), 17 nuthatches (*Sitta europaea*), and 7 coal tits (*Periparus ater*). About 97 % of the blue tits in the area use the available boxes for nesting. Blue tits can tolerate disturbance, which is important when it comes to handling and blood sampling. The life histories of the individuals were well known due to the long-term monitoring of the blue tit populations in the area, making it well suited for the present study on telomeres.

2.2 Data collection

The nest boxes were cleaned after the broods had fledged in 2016 and again in the beginning of spring (end of March 2017). The boxes were then checked every 2-3 days for signs of nest building. Ownership of the boxes was determined by observing the coloured leg bands of the birds. Following the nest building period, the nest boxes were checked frequently to monitor laying and hatching dates. To estimate the date of when the first egg was laid, I assumed that one egg was laid per day. During the incubation period, each nest was usually not visited until

the estimated time of hatching to avoid stressing the females unnecessarily. Monitoring continued after hatching to determine the final brood size.

Between May 5th and June 13th when most females were incubating or brooding, adults were caught close to their nest boxes using a mist net, playback of male song, and a caged male from another study area. A few males and females were also caught using nest clap traps. There was a bias towards capturing males and individuals caught in 2016, because there was an interest in comparing telomere lengths in blood (my research) and in sperm (Avery MacNeish's thesis, see below) of the same individuals from year to year. Local recruits were also targeted because of the information available on their exact age and rearing conditions. In total, blue tits from 41 nest boxes were captured, which made up 34 % of all the nest boxes occupied by blue tits in the area in 2017. Once captured, body mass was measured using a Pesola 50 g spring balance, tarsus length measured with a caliper and wing length with a wing ruler, and blood samples were collected. Immigrants were aged as one year or older based on their plumage colouration (Svensson, 1992). All measurements on adult birds were done by Arild Johnsen, to avoid inter observer measuring differences. Blood samples were stored in 2 ml microtubes containing Invitrogen™ RNAlater® Stabilization Solution (Thermo Fisher Scientific). The birds and their ring combination were photographed to secure correct identification. Within an hour of capturing, all birds were released in close proximity to their nest boxes.

Within the first three days after hatching, nestlings were counted and weighed with a Pesola 10 g spring balance. Hatching date was estimated from the body mass of the heaviest nestling with the use of a growth curve. When the oldest chick was 15 (± 1) days old, they were re-counted and weighed. Additionally, they were banded with an aluminium ring with a unique identification number. A blood sample was taken from the brachial vein from nestlings belonging to parents captured that same spring. Blood samples were stored in 2 ml microtubes with 1 ml 96 % ethanol. After fledging, the nest boxes were checked for remaining dead nestlings to determine the number of fledged chicks. The body mass of the successfully fledged young was used to obtain an estimated mean body mass for each brood. The rearing conditions were different in the two seasons, apparently with a higher availability of food in 2017 compared with 2016 (Tore Slagsvold, personal observation).

All samples were stored in a refrigerator at 4°C at the Natural History Museum (NHM), University of Oslo, before they were analyzed in the lab in autumn of 2017. All collected samples were registered in the Corema database of the NHM DNA bank.

2.3 Data set

This master thesis is a continuation of Ingvild Aabye's master thesis (Aabye, 2017). The samples she collected and analysed in 2016 were included to increase sample size. Accordingly, the laboratory protocol used by Ingvild Aabye was also used in this thesis. The Quantitative Real-time Polymerase Chain Reaction (qPCR) method is very sensitive and inter observer dependent. The samples from 2016 were therefore reanalysed in 2017 by Avery MacNeish (investigating telomeres in sperm and blood) and myself (investigating telomeres in blood), to be able to combine them with samples from 2017. All laboratory work (including DNA extraction) and field work was done in collaboration with Avery MacNeish. Of the 127 blood samples attempted to be analysed, two samples of the same individual from 2016 were removed from the analysis because they generated a wide range of different result the three times they were analysed with qPCR. One chick from 2016 was also removed because of the lack of a sample from adulthood, and 15 samples with mean quantification cycle (Cq) value > 0.208 were excluded (see statistic analyses). The final data set includes 109 individuals including 36 females, 63 males and 10 nestlings. Of these, 20 females, 32 males, and 10 nestlings were sampled in 2016, and 16 females and 31 males were sampled in 2017. Cross-sectional comparisons of 2016 and 2017 samples were done, but also longitudinal data from the two years were analysed.

2.4 Laboratory work

2.4.1 DNA extraction

DNA was extracted from blood samples using the E.Z.N.A.® Blood and Tissue DNA Kit (Omega bio-tek). The manufacturer's protocol was used with the following modifications: After thawing, 50 µl of the sample was mixed with 150 µl elution buffer to bring the volume up to 200 µl. Then 20 µl OB protease solution and 200 µl BL buffer were added before they were vortexed. The mixture was then incubated in the blood lysis buffer at 70°C for 30 min. To make the solution more viscous, 200 µl of 100 % ethanol was added. Afterwards, the entire sample was transferred to a HiBind® DNA mini column and centrifuged, then the filtrate was

discarded and 400 µl HBC buffer was added. After washing, the DNA was eluted from the HiBind ® DNA mini column using 100 µl elution buffer preheated to 70°C, making a final volume of 100 µl of extracted DNA.

DNA concentrations were measured using Invitrogen™ Qubit® 2.0 Fluorometer (ThermoFisher Scientific). Concentrations of DNA samples ranged from 1.3 to 67.3 ng/µl. DNA extractions were stored in the refrigerator at 4°C until qPCR analysis was conducted three weeks later.

2.4.2 Estimating the T/S-ratio with qPCR

The protocol used was optimized for blue tits by Angela Pauliny at the University of Gothenburg, based on a protocol developed by Cawthon (2002). qPCR can monitor the amplification of PCR products in real time by using a fluorescent double stranded DNA-binding dye. In other words, the relative yield of double stranded DNA is calculated in each cycle of the PCR reaction by monitoring the fluorescent signal. For each cycle, the amount of product will ideally double and accumulate exponentially until it reaches a stationary phase when all reaction components have been used. When the amount of amplified PCR products has built up, it reaches a threshold where the qPCR instrument can detect the emitted fluorescent signal, and the cycle for which this occurs is referred to as the quantification cycle (Cq) (Cawthon, 2002; Criscuolo *et al.*, 2009).

Overview of qPCR protocol

The concentration of extracted DNA was analysed using the Qubit the day before or the day of qPCR analysis. The samples were run on clear-well plates (Hard-Shell® 96-Well PCR Plates, Bio-Rad Laboratories Inc.) with adhesive seals (Microseal® 'B' Adhesive Seals, Bio-Rad) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For the amplifications, qPCR reagent SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) was used. Amplifications of the telomeres and the single-copy reference gene were run on different programs (table in Appendix 2). A melt curve analysis was done after each run to see that it had resulted in a specific PCR product. The assay is sensitive to differences in concentrations of both sample DNA and SYBR® Green Supermix, so extra care was taken to ensure equal DNA concentrations by diluting the samples to the same amount of DNA, and that the pipetting technique was as similar as possible between the wells and the plates. This was done by one individual (me) pipetting the Mastermix containing the SYBR® Green, and

one individual (Avery MacNeish) pipetting the sample. This was always performed in the same order; the mastermix pipetted first, and then the sample. All 162 samples were analysed on a total of 10 plates. Ideally the analysis of multiple plates should be done in a short time period to remain consistent, but due to schedule constraints it took two months.

Primers used for amplification of telomeres were Tel1b (forward) and Tel2b (reverse) (Criscuolo *et al.*, 2009). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single-copy reference gene and was amplified with primers GAPDH-F and GAPDH-R (Criscuolo *et al.*, 2009). These GAPDH primers are specific for zebra finches but were validated for blue tits by Angela Pauliny. Primers were diluted with purified Milli-Q H_2O from a stock, producing a final concentration of 3.5 µM for both forward and reverse GAPDH-primers, 2 µM for Tel1b and 4 µM for Tel2b.

Assessing amplification efficiency

To check the efficiency of the analysis, standard curves for telomeres and GAPDH reference genes were run separate from the amplified samples. One random DNA sample was used to create a 45 µl stock of 1 ng/µl (telomeres) and 2 ng/µl (GAPDH) and used as the basis for the serial dilution (1:3) for six replicates with six duplicates at each concentration (see Appendix 3). A master mix was prepared separately for each standard curve with 5 µl 1X SYBR® Green Supermix and 0.5 µl of forward and reverse primer per well. In total the wells contained a volume of 10 µl, 4 µl of serial diluted DNA and 6 µl master mix. A triplicate No Template Control (NTC) containing 10 µl Milli-Q H_2O instead of DNA was included on each plate to ensure that there was no contamination.

An optimal analysis should yield a linear standard curve ($R^2 > 0.980$) (Taylor *et al.*, 2010) and have an amplification efficiency (E) in the range of 85-115 % (Criscuolo *et al.*, 2009) (calculated as $E = 10^{[-1/slope]}$) (Pfaffl, 2001), with little variation among triplicates. An E value of 100 % would mean a perfect doubling of product in each qPCR cycle (Svec *et al.*, 2015). The qPCR analysis software CFX Maestro™ 3.1 (Bio-Rad) was used to generate the standard curves with R^2 and efficiency estimates. The telomere and GAPDH standard curves were run in three separate batches. Two standard curves were constructed one to two days before the first qPCR plates of samples were run, and one more standard curve was run one day after the 10th sample plate. This was done to assure that the amplification efficiency would be as accurate as possible. An estimate of the three standard curves for telomere and GAPDH was made by calculating the average E-value from the three standard curves. The average telomere

standard curves efficiency was 106.13 %, (E = 110.5 % and R^2 = 0.995; E = 106.2 % and R^2 = 0.996; E = 101.7 % and R^2 = 0.993). The average GAPDH standard curves efficiency was 99.67 %, (E = 97.2 % and R^2 = 0.994; E = 102.7 % and R^2 = 0.997; E = 99.1 % and R^2 = 0.994).

Amplification of sample

Extracted DNA samples were diluted to a 0.25 ng/µl working stock the same day as the amplification. The same working stock of DNA was used for both telomeres and GAPDH amplifications, which were amplified the same day on separate plates. For each plate, a master mix was prepared separately containing 5 µl 1X SYBR® Green Supermix and 0.5 µl of each primer (forward and revers) for each well. The total volume of each well was 10 µl, with 6 µl master mix and 4 µl (1 ng) sample. Each DNA sample was amplified in triplicates, and for the corresponding telomere- and GAPDH-plates, the same samples were positioned in the same well location. A triplicate NTC and three triplicate Inter Plate Calibrators (IPCs) were included on each plate, of which IPC2 was used as a reference sample when calculating the T/S-ratio, since this was the one that worked on all the plates.

2.5 Analysing the data

2.5.1 Interpreting results and calculating the T/S-ratio

The qPCR analysis software CFX Maestro™ (Bio-Rad) was used to collect and analyse data from the qPCR-analyses (see Appendix 4 for CFX Maestro™ output). The mean Cq value of the triplicates were calculated for each sample. Samples where the standard deviation of the mean Cq value was > 0.2, were re-analysed. There were three samples with values between 0.2 and 0.206 that were included because of their close proximity to 0.2, while 15 samples with the mean Cq value > 0.208 were excluded. The intra-plate coefficient of variation (CV %) between samples run in triplicates was on average 0.93 % for telomeres (ranging from 0.038 % to 1.86 %, with n = 185 in total on all 10 plates, including IPCs) and 0.29 % on average for GAPDH (ranging from 0.011 % to 0.74 %, with n = 185). The inter-plate coefficient of variation (between the ten plates) for IPC2 was on average 0.88 % for telomeres (0.31 % - 1.82 %) and 0.39 % for GAPDH (0.05 % - 0.70 %). The NTCs never had a fluorescent signal that reached above the baseline threshold set by the software.

The telomere-single-copy reference gene-ratio (T/S-ratio) is the number of copied telomeric repeats (T) relative to the number of copies of the single-copy reference gene (S) (Cawthon, 2002). The relative T/S-ratio was calculated using Pfaffl's method (Pfaffl, 2001), in the following equation:

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

CFX Maestro™ calculated the amplification efficiency estimate of the analysis from the telomere and GAPDH standard curves, before it was converted into an E factor, E = (E % /100) + 1. The mean Cq-value of each sample was subtracted from the mean Cq-value of a reference sample (IPC2) to obtain ΔCq target (telomeres) and ΔCq reference (GAPDH). This relative difference between individuals should reflect the relative telomere length of their DNA (Cawthon, 2002), and serves as the basis for all further statistical analyses.

2.5.2 Statistical analyses

All statistical analyses and graphic illustrations were generated using R 3.3.1 (R Core Team, 2016), except the normal distribution that was checked using SPSS (IBM, SPSS Statistics 25), using Shapiro-Wilk tests and visual inspection of QQ-plots. To dismiss the null hypothesis (H0), a significance level of 0.05 was used.

The T/S-ratio, from now on referred to as relative telomere length (rTL), was normally distributed when log transformed ($p = 0.213$) for all adult individuals. The log of rTL is therefore used in all analyses. Relative telomere length for the ten nestlings was not normally distributed, not even after log transformation ($p = 0.036$).

In the following analyses, rTL was the response variable when predictions of rearing conditions and morphology were investigated. When investigating reproductive success, the fitness-measurements were used as response variables. For reproductive success, parents who lost their entire brood after hatching were excluded, as the cause was often hard to establish, and not necessarily related to the parents' quality. Pearson or Spearman correlations are used for all analyses, since the model assumptions for the linear mixed models (LMM) was not

met in most of the cases (table of correlations in Appendix 6). The model assumptions of the LMMs were checked by visual inspection of QQ-plots and homoscedasticity plots. Year and collection date were included in the LMMs as they proved to be significant (see results), and individual ID (ring number) was included as a random factor to account for repeated samples of the same individual from the two years. Reductions of the LMMs were done with a stepwise backwards reduction. Seven of the individuals were caught in both 2016 and 2017, and these were treated as independent datapoints. Potential pseudoreplication of these seven individuals was checked by choosing one of the two data points from the two years at random, which gave qualitatively similar results. Differential survival was checked with a t-test and a logistic regression (survived/not survived as response variable), using rTL from 2016. In addition to the seven adult individuals caught both years, 10 nestlings from 2016, were caught as one-year-old's in 2017. I tested whether rTL differed between the years, using a Wilcoxon matched pairs signed rank test. In the figures, regression lines are added for illustrative purposes, even when there was no significance.

2.6 Ethical note

During the incubation period, females were avoided or released immediately if captured to avoid nest desertion. The nests were not visited every day to reduce disturbance, or if it was too cold to reduce cold stress. Only a small amount of blood (< 25 µl) was taken from the birds, and there was no sign that blood sampling affected their immediate survival. Authorization to collect blood samples was given by the Norwegian Food Safety Authority (Mattilsynet), and authorization for ringing and mist net catching was given by the Norwegian Environment Agency (Miljødirektoratet).

3 Results

3.1 Telomere length in relation to year, collection date, age and sex

Telomeres were on average 17 % longer in 2017 (n = 47) than in 2016 (n = 52) (t-test, t = 3.66, p < 0.001). Separating by sex, the year differences was not significant for females (t = 1.77, n = 36, p = 0.091; Figure 1a), but telomeres were significantly longer in 2017 for males (t = 3.23, n = 63, p = 0.003; Figure 1b). Telomere length for both years increased with collection date (Spearman correlation, r_s = 0.22, n = 99, p = 0.027; Figure 2). In 2017, more adult blue tits were captured later than in 2016; however, when including collection date in a LMM (table in Appendix 5), the effect of year was marginally significant (p = 0.077) whereas the effect of collection date was still significant (p = 0.013). Year differences did not explain the significance of collection date, as the interaction between the two was not significant (p = 0.35).

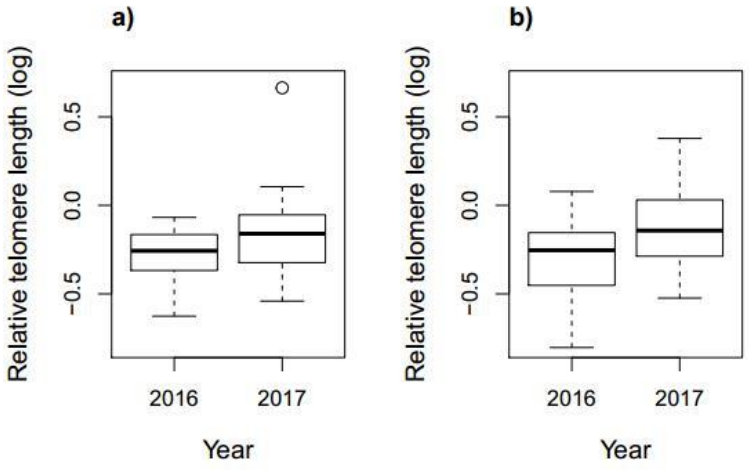

Figure 1. Box plot showing the difference between years in relative telomere length for a) adult female (2016: n = 20; 2017: n = 16), and b) adult male blue tits (2016: n = 32; 2017: n = 31).

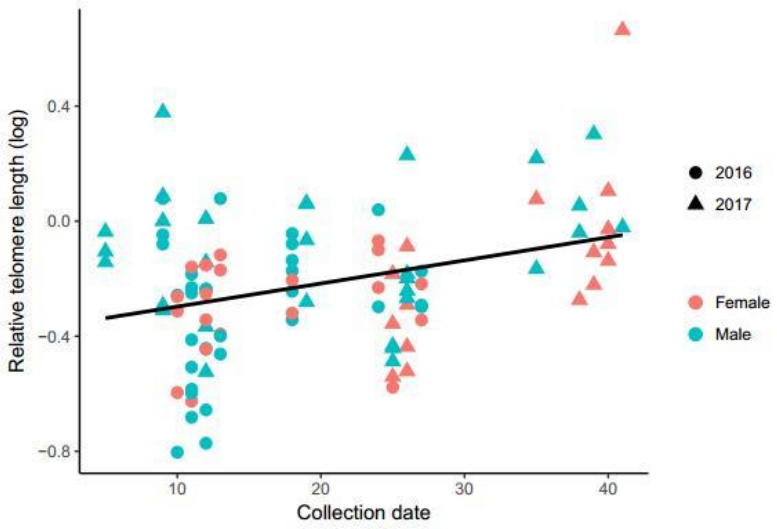

Figure 2. Relative telomere length for 99 adult blue tits, 36 females (20 from 2016 and 16 from 2017), and 63 males (32 from 2016 and 31 from 2017), in relation to collection date. 1 = 1th of May. A regression line is shown for the combined data.

Longitudinal comparisons of the 17 individuals caught both years did not yield any significant patterns. There was no systematic change in telomere length from the nestling to the adult stage (one-year-old) (Wilcoxon matched pairs signed rank test, V = 20, n = 10, p = 0.49; Figure 3). The seven adult individuals captured in both 2016 and 2017, did not show any significant change in rTL from one year to the next (V = 17, n = 7, p = 0.69; Figure 4). In cross-sectional analyses, there was no significant correlation between rTL and age for all individuals combined (r = -0.12, n = 97, p = 0.2497), nor for females (r = -0.23, n = 35, p = 0.18) or males (r = -0.08, n = 62, p = 0.55) analysed separately (Figure 5). There was no significant difference in telomere length between females and males (t = 0.37, p = 0.71).

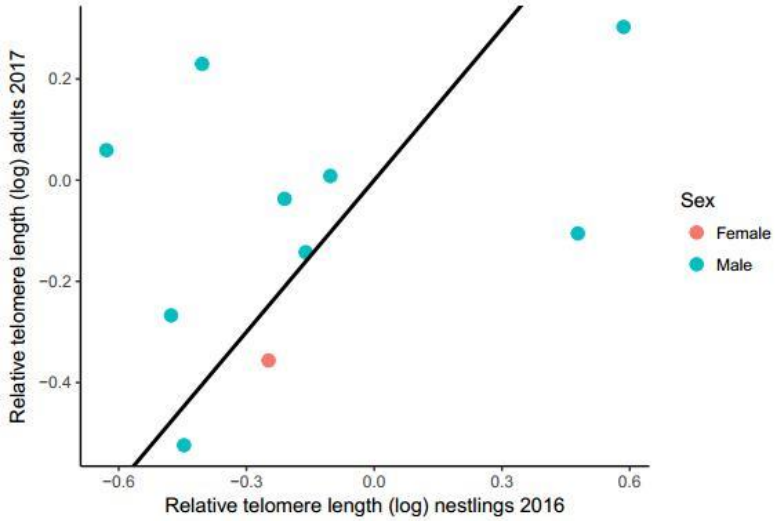

Figure 3. Relationship between the relative telomere length of nestling blue tits from 2016 caught as one-year-old's in 2017 (n = 10, 1 female and 9 males). Individuals above the black line of unity showed an increase, while the individuals under the line showed a decrease in their relative telomere length over the course of one year.

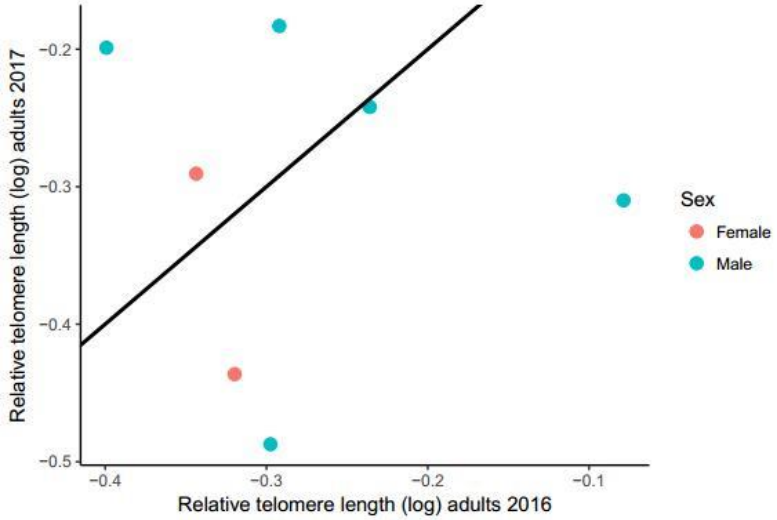

Figure 4. Relationship between the relative telomere length of adults from 2016 caught again as adults in 2017 (n = 7, 2 females and 5 males). Individuals above the black line of unity showed an increase, while the individuals under the line showed a decrease in their relative telomere length over the course of one year.

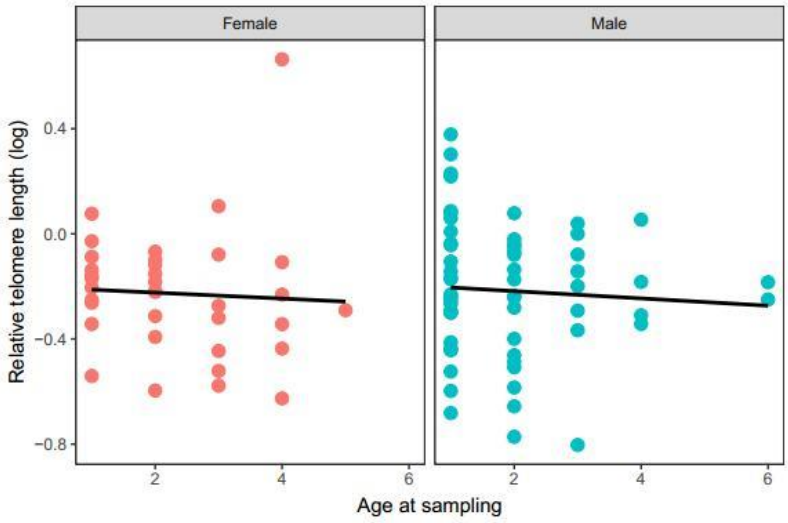

Figure 5. Relative telomere length related to the individual's age at sampling time, for female (red, n = 35) and male (blue, n = 62) blue tits.

3.2 The silver spoon effect (rearing condition)

Adults that had fledged from large broods had shorter telomeres than those that had fledged from small broods, when taking year and collection date into account in a LMM (Table 1). The correlation did not hold for females alone, but it did for males (Figure 6; Appendix 6). Relative telomere length of adult blue tits was not related to hatching date in the year they were born ($p > 0.52$), nor by its nestling body mass at 15 days old ($p > 0.33$). Results were similar when analysing the sexes separately (Appendix 6).

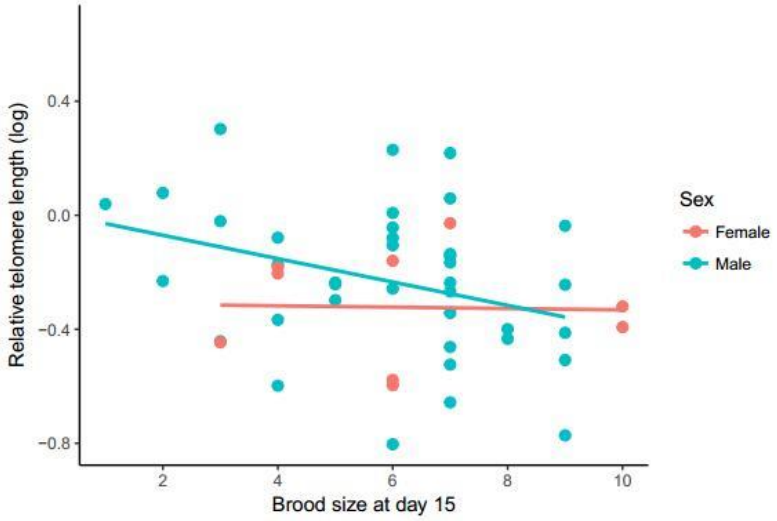

Figure 6. Relative telomere length in relation to brood size at day 15 for both years combined (female n = 9, male n= 38). Regression lines shown for each sex separately.

Table 1. The final linear mixed model for all adult blue tits with relative telomere length in relation to brood size, collection date and year.

	Estimate	SE	df	t-value	Pr (>|t|)
(Intercept)	-3.180	1.153	43	-2.76	0.009
Brood size	-0.030	0.015	43	-2.03	0.048
Collection date	0.006	0.004	43	1.64	0.11
Year	0.183	0.073	43	2.53	0.015

3.3 Individual quality (reproductive success)

For reproductive success, the sexes were analysed separately to avoid pseudoreplication, because the dataset contained 14 social pairs. Telomere length did not predict clutch size for females ($p > 0.75$), but males with longer telomeres had larger clutches ($p = 0.022$; Figure 7; Appendix 6). This was significant for 2016 ($p = 0.0014$), but not for 2017 ($p = 0.74$). Laying date was not predicted by telomere length for either sex with the two years combined ($p > 0.27$), nor when the two years were separated ($p > 0.11$). Of the adult blue tits, telomere length did not predict hatching date ($p > 0.091$), number of hatched eggs ($p > 0.20$), nor brood size ($p > 0.06$) for either sex (Appendix 6).

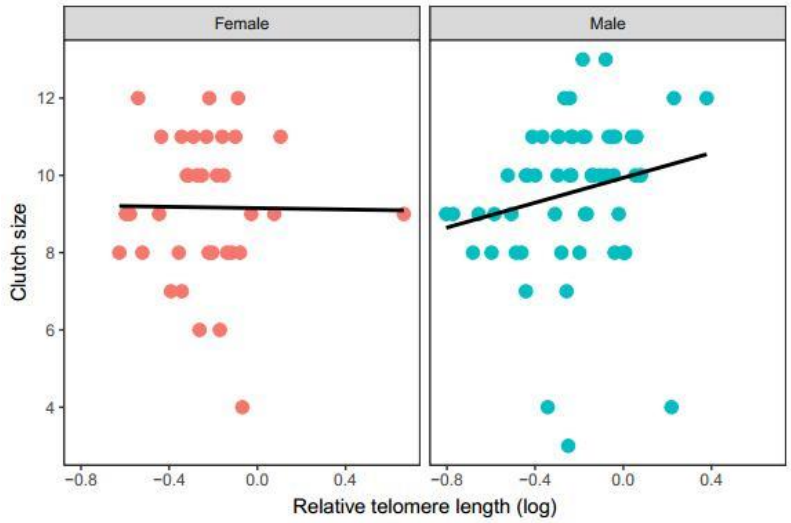

Figure 7. Relative telomere length in relation to clutch size with both years combined for female (red, n = 35) and male (blue, n = 58) blue tits.

3.4 Morphology

For analyses of morphology, females and males were separated because of sexual size differences. Female wing length was not significantly related to rTL ($p = 0.80$) (Appendix 6), but male wing length was shorter for individuals with longer telomeres ($p = 0.029$) (Figure 8), this held for 2017 but not for 2016 (2016 males: $p = 0.33$; 2017 males: $p = 0.02$). This relationship was also significant when collection date and year were included in a LMM analysis (Table 2). One-year-old males with longer wings had shorter telomeres ($p = 0.019$), whereas there was no significant relationship for older males from two to six years of age ($p = 0.52$) (Figure 9). Telomere length was not related to body mass at time of capture ($p > 0.18$) or tarsus length ($p > 0.46$) for either sex. This was also the case when analysing the data for each year separately (Appendix 6).

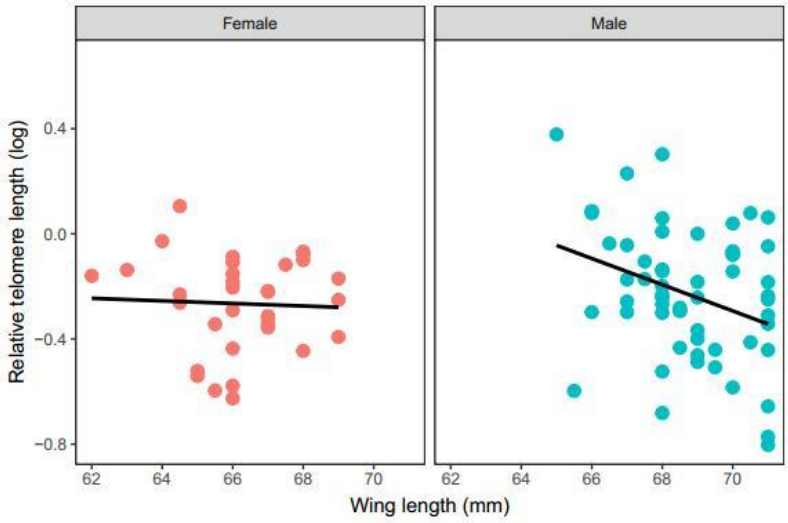

Figure 8. Relative telomere length in relation to wing length (mm) (both years combined), for female (red, n = 33) and male (blue, n = 57) blue tits.

Table 2. Linear mixed model of relative telomere length for male blue tits in relation to wing length, collection date and year.

	Estimate	SE	df	t-value	Pr(>|t|)
(intercept)	2.21	1.46	10	1.52	0.16
Wing mm	-0.05	0.02	36.43	-2.98	0.005
Collection date	0.006	0.004	30.36	1.57	0.13
Year	0.07	0.05	6.7	1.51	0.18

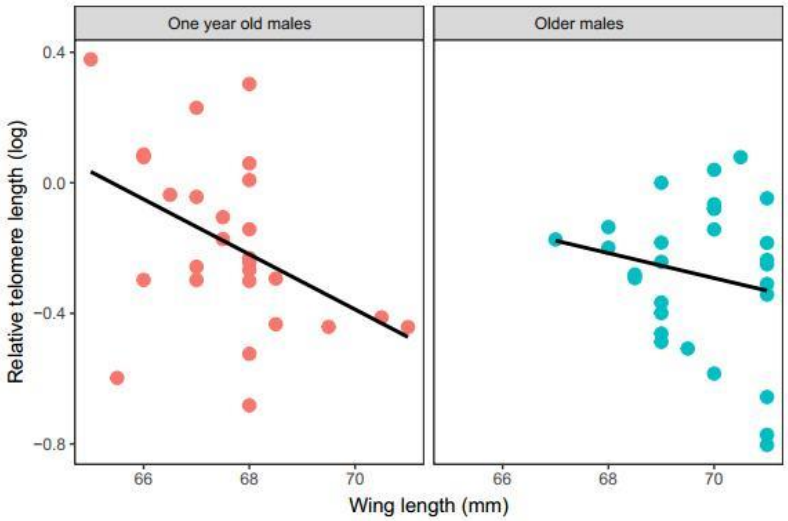

Figure 9. Relative telomere length in relation to wing length of young male blue tits (red, n = 27) and older male blue tits (blue, n = 29).

3.5 Survival

Of the 52 adult blue tits that were present in 2016, 24 were observed in 2017, which amounted to a survival rate of at least 46 %. Adult birds in this population that evidently survived from 2016 to 2017 did not have longer telomeres than those that did not appear in 2017 (t = -0.27, n = 52, p = 0.79) (Figure 10). The results were similar when age at sampling, sex, and collection date were included in a multivariable logistic regression model (Table 3).

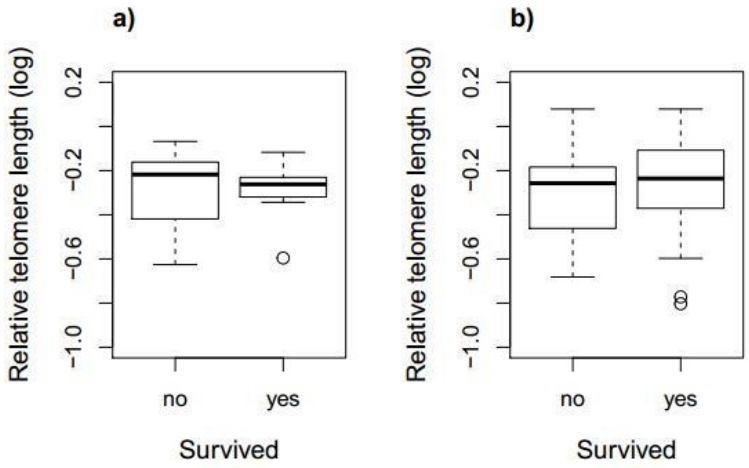

Figure 10. Box plot showing relative telomere length in relation to survival for a) female (n=20) and b) male (n=32) blue tits.

Table 3. Multivariable logistic regression model of survival for adult blue tits.

	Estimate	Std. Error	z-value	Pr(<|z|)
(intercept)	-0.66	1.43	-0.46	0.65
Log(rTL)	0.22	1.38	0.16	0.88
Collection date	0.02	0.05	0.50	0.62
Sex	0.12	0.58	0.20	0.84

4 Discussion

In this study of the blue tit, I analysed telomere length in relation to a number of factors. The silver spoon hypothesis was supported by the fact that telomere length of the individual as an adult seemed to reflect its rearing condition as a nestling, with those growing up in larger broods having shorter telomeres as adults than those growing up in smaller broods. The individual quality hypothesis was supported by the fact that males with longer telomeres had mated with females that laid larger clutches. However, the hypothesis was not supported by the finding that males with longer wings had shorter telomeres (mainly first year birds). In addition, I found that individuals caught in 2017 had longer relative telomere length than individuals caught in 2016, and individuals caught later in the season had longer telomeres than those caught earlier. Some of the individuals caught both years showed an increase in rTL, others showed a decline.

My findings suggest that adults fledged from larger broods have shorter telomeres than those fledged from smaller broods. This supports the silver spoon effect hypothesis and is validated by several studies finding a correlation between enlarged broods and shorter telomeres at the nestling stage (Boonekamp *et al.*, 2014; Watson *et al.*, 2015; Young *et al.*, 2017). The survival rate of blue tit nestlings from enlarged broods was also found to be decreased compared to those broods that were reduced in size (Råberg *et al.*, 2005). One reason could be that growing up in a larger brood can lead to more exposure to oxidative stress that shortens telomeres (Watson *et al.*, 2015). The amount of food given to individual nestlings also decreases with increasing brood size. Although parents may increase their feeding rate when their brood is enlarged, it is not proportional to nestling numbers (Saino *et al.*, 2000). Large brood size may also result in a larger number of nest-dwelling parasites (Saino *et al.*, 2002). Factors like crowded broods, oxidative stress, competition for food, and increased number of parasites may, either combined or alone, shorten the nestling's telomere length.

In this population of blue tits, males with longer telomeres had mated with females that laid larger clutches. Females have been shown to adjust egg size and number in response to male attributes (Cunningham, 2000; Horváthová *et al.*, 2012). Bird species where the female solely take care of the chicks tend to lay larger eggs (Cunningham, 2000), while birds with bi-parental care have been shown to lay more eggs when mating with a high-quality male (Horváthová *et al.*, 2012). Increased investment when mated to a high-quality male may be favoured by selection if it increases female fitness, a phenomenon termed differential allocation (Burley, 1986). Since both the male and female blue tits feed their young, males

with long telomeres might signal good parental qualities, for instance through efficient foraging activities, or through their plumage colouration (Andersson *et al.*, 1998). Alternatively, males with longer telomeres might attract high-quality females that lay large clutches. My results indicate a connection between telomere length and male quality but they cannot distinguish between these alternatives.

I found a negative correlation between rTL and wing length for male blue tits (mainly first year birds). Several studies show the opposite, that long telomeres in fledglings are positively correlated with wing length (Nettle *et al.*, 2013; Parolini *et al.*, 2015; Young *et al.*, 2017). However, fledglings do not have fully grown wings (Flegg & Cox, 1977; Garnett, 1981) and the birds in these studies were not measured as adults. To my knowledge, few studies have looked at, or found a relationship between rTL and morphological traits in adult birds. Johnsen *et al.* (2017) investigated a number of different morphological traits in relation to rTL in adult bluethroats (*Luscinia svecica*) but found no significant relationships. Similarly, I found no significant correlation among older males between wing length and rTL, but one-year-old males with short telomeres had longer wings. In blue tits, first year birds have shorter wings than older birds (Flegg & Cox, 1977). We captured the adult blue tits during the breeding season, so any first-year birds we caught still had their nestling feathers, as the feathers are fully grown by late August and are molted once a year at the end of the breeding season (Flegg & Cox, 1977; Svensson & Nilsen, 1997). A negative relationship between rTL and morphological traits could arise because the physiological cost of growing to a big size causes telomere shortening (Costanzo *et al.*, 2017). Hall *et al.* (2004) found that European shag nestlings (*Phalacrocorax aristotelis*) with rapid growth had higher telomere erosion than nestlings with normal growth rates. This could explain why individuals with longer wings have shorter telomeres. I did not find a significant correlation between rearing conditions and adult wing length (data not shown), hence this seems to be unrelated to the silver spoon effect and is probably more related to individual quality. The cost of growing wings to a certain length can have a toll on rTL (Costanzo *et al.*, 2017).

The blue tits had longer rTL in 2017 than in 2016, a similar year effect was found in a population of bluethroats (Johnsen *et al.*, 2017). This may have been caused by different levels of stress experienced by birds sampled in the two years, e.g. during the preceding winter and/or breeding season, but I do not have the data to test this. In both years, telomere length increased with collection date. Late captured individuals had longer telomeres than individuals captured early. A cross-sectional study on the common tern (*Sterna hirundo*)

showed that independent of age, individuals with shorter telomeres arrived earlier, reproduced earlier, and had more nestlings (Bauch *et al.*, 2013). Bauch *et al.* (2013) hypothesized that adults that are successful in one year, have been successful in the past, which has shortened their telomere lengths in comparison to adults that are less successful with brood rearing. In other words, there is a cost of reproduction. Another explanation could be terminal investment, with individuals arriving earlier being older and having shorter telomeres, and hence investing more in reproduction. Even though I found no relationship between rTL and age in this blue tit population (see below), I cannot rule out the possibility that a larger sample size would reveal such a pattern. The findings that blue tits had longer telomeres in 2017 than in 2016, and that rTL increased with collection date are interesting, but at present I can not explain these relationships.

Ten individuals sampled as nestlings in 2016 were sampled again as adults in 2017. Telomere length had increased in six individuals and decreased in four individuals. Increase in rTL in some nestlings has been found in one other passerine bird, the barn swallow (Parolini *et al.*, 2015). Parolini *et al.* (2015) speculated that the variation could be due to individual genetic differences or because of the biochemical composition of the egg and is therefore caused by early maternal effects. On the other hand, a cross-sectional study of bluethroats found an overall reduction of rTL during the nestling phase (Johnsen *et al.*, 2017), which has also been found in a longitudinal study of jackdaws (Boonekamp *et al.*, 2014). The results are inconclusive, and more data is needed.

Seven adult individuals were caught in both seasons and rLT had increased in three individuals and decreased in four individuals. For the cross-sectional analysis, there was no significant relationship between rTL and individual age. Age ranged from one to six years but there were few individuals in the older age classes, which reduces the statistical power of the test. In a study on adult alpine swifts (*Tachymarptis melba*), two out of 22 individuals showed an increase in rTL over the course of five years (Bize *et al.*, 2009). There is no clear reason why some individuals have an increase in rTL over time, as telomerase activity is inactive in most post-natal somatic tissues (Haussmann *et al.*, 2007). Some long-lived bird species, such as the common tern and the Leach's storm petrels (*Oceanodroma leucorhoa*) have been shown to have a high level of bone marrow telomerase activity throughout their lives, which can lengthen telomeres (Haussmann *et al.*, 2007). Sudyka *et al.* (2016) investigated longitudinal data on blue tits and found that they have one of the highest rates of telomere erosion found in birds. Since the blue tit has a short lifespan with a high risk of predation,

starvation, and accidents (Tricola *et al.*, 2018), a larger sample size will be needed to show a cross-sectional relationship between age and rTL. Whether the discrepancy between my results and those of Sudyka *et al.* (2016) reflect real population differences or a lack of statistical power in my dataset is currently unknown.

Blue tits that survived from one year to the next as adults, did not seem to have longer telomeres than those that apparently did not survive. Haussmann *et al.* (2005) found that longer telomeres were associated with a higher survival rate in the tree swallows (*Tachycineta bicolor*), while in a study on barn swallows, Caprioli *et al.* (2013) found no relationship between rTL and lifespan. As with age, the reason could be that individuals are disappearing from the population regardless of their rTL due to predation and other external factors (Tricola *et al.*, 2018). Another reason could be that the rate of telomere change varies over time. There is often a high rate of rTL change early in life that gradually decrease before it accelerates again shortly before death (Salomons *et al.*, 2009). By measuring rTL at the same time point each year, one might miss the crucial point of telomere erosion that happens before death. This study only investigated survival over the course of one year. If the same population is followed for a longer time period, then it might be possible to find a relationship between rTL and survival rate.

No difference was found between the sexes in their rTLs. In blue tits, males typically have a longer lifespan than females, which may be due to their social dominance over food resources during winter (Hansen & Slagsvold, 2004). Male blue tits could therefore be expected to have, on average, longer telomeres than females of the same age. In humans, females live, on average longer than males, and they have, on average, longer telomeres (Bakaysa *et al.*, 2007). A study on the lesser black-backed gull showed that males tend to have longer telomeres as hatchlings than females (Foote *et al.*, 2011). My results show that telomere length does not differ between the sexes in the investigated blue tit population, which is a relatively common pattern in birds (Barrett & Richardson, 2011), and supported by findings on a population of blue tits in Gotland, Sweden (Sudyka *et al.*, 2014).

The sample sizes in this study are small for a number of my analyses. A larger sample size and a more equal representation of the sexes could have generated different results. Some of the p-values were marginally significant and would not have been significant with correction for multiple testing. I still choose to interpret the p-values as being biologically meaningful, as they might give indications of patterns that would be more highly significant with a larger

dataset, and thus worth following up in future studies. The qPCR method used is sensitive to systematic differences when carried out by different individuals or at different time points (e.g. conditions in the lab, changes in hardware and chemicals, and pipetting technique). As such, samples to be compared should be analysed by the same individual, at one time period. To reduce systematic error, data from 2016, sampled and analysed by Ingvild Aabye, was reanalysed by Avery MacNeish and myself to be able to compare them to samples from 2017.

5 Conclusion

This study indicates that there is a silver spoon effect on telomere length in blue tits, as individuals growing up in larger broods had shorter telomeres as adults. More longitudinal data, over the course of several years, should be collected to test if this is a representative and long-lasting effect. Telomere length may also reflect individual quality, as we found that male blue tits with longer telomeres mate with females that lay larger clutches. However, this was contradicted by the finding that male blue tits with longer wings had shorter relative telomere length. More research is clearly required to disentangle the relationships between rearing condition, individual quality, and relative telomere length in blue tits.

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Appendix

1) Telomeres

Telomeres end in a single-strand overhang at the 3' end that folds back over the duplex DNA to form a telomere-loop (t-loop) structure. The t-loop protects the telomeric ends from fusing by preventing DNA ligase from fastening (De Lange, 2004). Telomeres play a role in preventing chromosome degradation and fusion (Blackburn, 1991). Telomeres shortens with each cell division due to the inability of DNA polymerase to completely replicate the end of the new lagging strand, referred to as the end replication problem (Watson, 1972). DNA polymerase operates in a 5' to 3' direction on double-stranded DNA and requires RNA primers for polymerase to bind and function. The leading strand is replicated in full, but the lagging strand is replicated discontinuously and requires multiple primers. At the end of the process polymerase replaces the RNA primers with DNA. However, at the end of the lagging strand there are no double stranded region for the polymerase to attach to, so the RNA is not replaced with DNA. The lagging strand loses the DNA sequence where the last RNA primer was laid down in each cell division. (Levy *et al.*, 1992; Haussmann & Marchetto, 2010). In this way the presence of telomeres acts as a buffer and protects the genes against degradation.

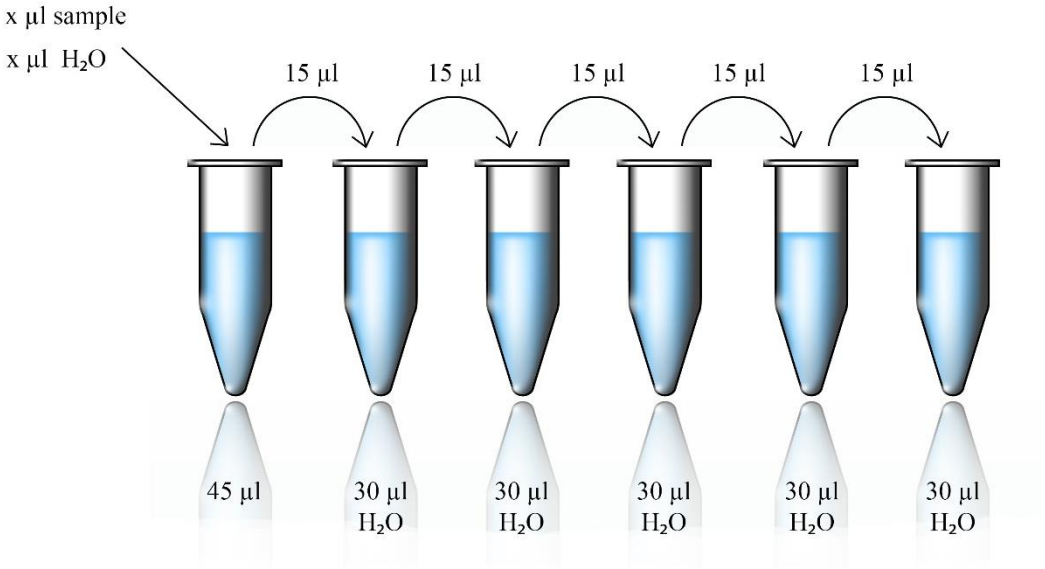
2) The qPCR program

Table showing the qPCR programs for telomere- and GAPDH-amplification, with 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)	59 - 96°C (0.5 increase cycle)
Hold	15°C	15°C

3) Serial dilution for standard curves

One DNA sample was used to create a 45µl stock of 1 ng/µl (telomeres) and 2 ng/µl (GAPDH) and used as the basis for the serial dilution (1:3) for six replicates with six duplicates at each concentration. How many µl of sample and H_2O added in the first tube is dependent of the DNA concentration of the sample, as the final volume of 1 ng/µl (telomere) or 2 ng/µl (GAPDH) should amount to 45 µl, and thus varies from standard curve to standard curve.



Telomere	1 ng/µl	0.33	0.11	0.037	0.012	0.004
GAPDH	2 ng/µl	0.67	0.22	0.074	0.024	0.008

4) Output from CFX™ Maestro [next page] showing all samples included in the study and on which plate they were run. All samples were run in one of ten sessions, and samples with a standard deviation above 0.2 (rule of thumb) was run again on plate eight, nine or ten. Samples that still had a standard deviation above 0.2 were excluded from the dataset (not shown). Each run included two plates, one for amplification with telomere-primers and one with GAPDH-primers. All samples and IPCs were run in triplicates on each plate, and the mean quantification cycle (Cq) was calculated by the program. IPC2 is sample 91451 and marked in yellow.

Plate 1				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10.2	0.032	26.58	0.028
93151	10.61	0.153	26.86	0.062
91459	10.81	0.028	27.23	0.05
93158	10.76	0.127	26.52	0.031
91456	11.45	0.172	27.13	0.061
93182	11.13	0.063	26.96	0.019
93196	11.28	0.05	27.02	0.047
91479	11.21	0.132	27.25	0.041
93290	10.69	0.188	26.86	0.03
91445	10.25	0.052	26.48	0.083
93314	10.51	0.152	26.32	0.063
91482	10.37	0.153	27.09	0.075
93321	9.84	0.05	27.05	0.069
91487	10.25	0.031	27.07	0.031
93328	11.05	0.089	26.56	0.027
91461	10.51	0.155	26.99	0.095
93345	10.73	0.143	26.83	0.038
91444	10.92	0.135	27.28	0.064
91449	9.83	0.031	26.74	0.065
91452	10.73	0.092	27.26	0.037

Plate 2				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10,83	0,144	27,74	0,155
86066	11.14	0.136	27.2	0.052
86085	10.91	0.133	27.71	0.206
86101	11.27	0.081	27.77	0.071
91465	11.15	0.158	27.37	0.012
86106	10.88	0.082	27.37	0.069
91474	10.5	0.08	27.13	0.018
86075	10.64	0.052	27.2	0.064
91481	10.83	0.034	27.39	0.006
91457	10.62	0.03	26.99	0.111
91458	10.54	0.186	27.23	0.11
91463	10.29	0.033	27.08	0.088
91470	10.76	0.099	27.04	0.026

Plate 3				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10,54	0,069	27,58	0,151
93239	10.08	0.1	26.74	0.034
91466	10.22	0.086	26.73	0.051
86105	9.82	0.113	26.33	0.052
91472	10.11	0.1	26.71	0.088
86088	9.94	0.142	26.49	0.022
91473	9.91	0.076	26.29	0.052
86053	9.93	0.076	26.08	0.042
86056	10.09	0.044	26.73	0.044
86058	10.16	0.142	26.73	0.098
86062	9.69	0.112	26.46	0.044
86065	10.22	0.065	26.34	0.035
86068	9.91	0.129	26.7	0.117
86072	9.8	0.101	26.31	0.033
86074	9.75	0.115	26.39	0.028
86076	9.77	0.03	26.13	0.031
86079	9.8	0.057	26.56	0.036
86081	9.88	0.071	26.72	0.011
86082	10.26	0.184	26.72	0.116
86092	10.01	0.054	26.73	0.019
86094	10.72	0.033	27.67	0.1
86095	9.97	0.066	26.65	0.108
86097	9.55	0.073	26.4	0.07
86100	10.44	0.073	26.64	0.09
86104	10	0.18	26.7	0.181

Plate 4				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	12,18	0,042	27,85	0,051
91468	11.63	0.086	27.01	0.105
91477	11.84	0.169	26.74	0.099
91489	11.75	0.204	27.36	0.09
91490	11.48	0.199	27.27	0.115
91491	11.62	0.181	27.15	0.063
91493	11.72	0.144	27.17	0.105
91932	10.86	0.014	26.58	0.116
91930	10.75	0.158	26.67	0.178
91931	11.09	0.126	26.47	0.072
91938	11.46	0.164	27.04	0.028
91939	10.7	0.064	26.38	0.034
91941	11.31	0.117	26.91	0.084
86049	11.21	0.082	26.72	0.009
86050	11.02	0.133	26.75	0.169
86051	11.28	0.165	26.84	0.083

Plate 5				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10,93	0,109	27,25	0,013
86057	11.4	0.074	26.58	0.127
86059	11.26	0.05	26.75	0.09
86060	10.81	0.048	26.39	0.046
86061	10.63	0.072	26.67	0.036
86063	10.57	0.086	26.54	0.042
86064	10.85	0.047	26.57	0.055
86067	11.06	0.157	26.4	0.075
86070	10.32	0.01	26.27	0.145
86073	10.91	0.186	26.28	0.06
86077	11.29	0.027	26.51	0.061
86080	11.1	0.027	26.76	0.039
86083	10.9	0.076	26.97	0.112
86087	11.05	0.032	26.88	0.043
86090	10.98	0.11	26.95	0.062
86091	11.13	0.084	27.21	0.014
86098	10.73	0.076	26.61	0.108
86099	10.34	0.03	26.69	0.052
86103	10.74	0.073	26.8	0.157

Plate 8				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	11,02	0,201	27,55	0,178
91446	11.15	0.056	27.48	0.088
93313	10.12	0.055	27.3	0.077
91450	11.34	0.096	27.45	0.108
91448	10.98	0.106	27.06	0.048
86086	11.07	0.063	27.54	0.037
86089	11.16	0.147	27.5	0.178

Plate 9				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10,72	0,082	27,42	0,111
86078	11.53	0.09	27.69	0.094
91464	12.23	0.181	29.09	0.02
91467	11.63	0.058	27.59	0.169
91476	10.86	0.046	27.44	0.07
91483	11.28	0.107	27.85	0.132
91484	11.14	0.084	27.54	0.087
91940	11.28	0.04	27.61	0.046
91942	11.09	0.078	27.24	0.037
86052	11.91	0.184	27.64	0.105
86069	11.18	0.006	27.54	0.095
86071	11.57	0.153	27.67	0.033
86084	10.86	0.037	27.68	0.051

Plate 10				
	Telomere		GAPDH	
Samples	Mean Cq	St.dev	Mean Cq	St.dev
91451	10,89	0,044	27,34	0,100
91453	11.42	0.162	27.47	0.061
91480	10.84	0.172	27	0.077
91460	11.24	0.117	27.3	0.063
91469	11.72	0.177	27.57	0.138
86055	11.15	0.099	27.24	0.154

5) Final linear mixed model for relative telomere length against collection date and year in this population of adult blue tits.

	Estimate	SE	df	t-value	Pr (>|t|)
(Intercept)	-1.752	0.746	25.2	-2.35	0.027
Collection date	0.006	0.002	92.0	2.54	0.013
Year	0.086	0.046	25.5	1.85	0.077

6) Summary of correlations between relative telomere length and the different traits examined in the blue tit population. Pearson correlations were used when both variables were normally distributed, and Spearman correlations were used when one variable was not normally distributed. a = Pearson correlation, b = Spearman correlation.

Hypotheses	Variable	Year	Female			Male			All		
			r	n	p	r	n	p	r	n	p
The silver spoon (rearing condition)	Hatching date (in birth year)	2016 & 2017	0.25[a]	9	0.52	-0.09[b]	38	0.60	-0.05[a]	47	0.73
	Brood size	2016 & 2017	-0.03[a]	9	0.94	-0.35[b]	38	0.03*	-0.28[a]	47	0.053
	Nestling body mass (mg)	2016 & 2017	0.27[a]	9	0.49	-0.16[b]	38	0.33	0.03[a]	47	0.85
Morphology	Tarsus length (mm)	2016 & 2017	-0.13[a]	33	0.48	-0.10[a]	57	0.46			
		2016	-0.24[a]	20	0.31	0.02[a]	31	0.90			
		2017	-0.07[a]	13	0.83	-0.07[a]	26	0.73			
	Wing length (mm)	2016 & 2017	-0.04[a]	33	0.80	-0.29[b]	57	0.03*			
		2016	0.09[a]	20	0.70	-0.18[b]	31	0.33			
		2017	-0.11[a]	13	0.71	-0.44[a]	26	0.02*			
		One year olds				-0.45[b]	27	0.019*			
		Older than one year				-0.12[b]	29	0.5242			
	Adult body mass (g)	2016 & 2017	-0.24[a]	33	0.18	-0.10[b]	57	0.47			
		2016	-0.36[a]	20	0.12	-0.04[b]	31	0.85			
		2017	-0.02[a]	13	0.95	-0.17[a]	26	0.42			

Hypotheses	Variable	Year	Female			Male			All		
			r	n	p	r	n	p	r	n	p
Individual quality (reproductive success)	Laying date	2016 & 2017	0.20[a]	35	0.27	0.02[b]	59	0.88			
		2016	-0.24[a]	20	0.30	-0.13[b]	32	0.48			
		2017	0.43[b]	15	0.11	0.75[a]	27	0.75			
	Hatching date	2016 & 2017	0.29[a]	35	0.091	0.04[a]	56	0.75			
		2016	-0.16[a]	20	0.49	-0.24[a]	31	0.20			
		2017	0.44[b]	15	0.10	0.21[a]	25	0.31			
	Clutch size	2016 & 2017	-0.01[a]	35	0.95	0.30[b]	58	0.02*			
		2016	-0.03[a]	20	0.90	0.54[b]	32	0.0014***			
		2017	-0.10[b]	15	0.75	0.07[b]	26	0.74			
	Number hatched eggs	2016 & 2017	-0.02[a]	35	0.90	0.07[b]	58	0.62			
		2016	-0.30[a]	20	0.20	0.09[a]	31	0.65			
		2017	-0.03[a]	15	0.93	-0.13[a]	27	0.53			
	Brood size	2016 & 2017	0.06[a]	33	0.76	0.01[b]	50	0.95			
		2016	0.10[a]	19	0.69	0.16[a]	26	0.43			
		2017	-0.10[a]	14	0.73	-0.39[a]	24	0.061			

7) Complete data set

The complete data set [the next pages]. Brood size was counted at day 15 and the number of dead nestlings discovered in the nest after that was subtracted.

Sample	Collection date	Was chick	Age at sampling (minimum)	Sex	Survived	Body mass (g)	Tarsus length (mm)	Wing length (mm)	Relative telomere length	Plate (PCR amplification)	Hatching date	Body mass (g) 15 days	Brood size	Laying date (1 = 1 April)	Clutch size	Hatching date (1 = 1 May)	Number hatched eggs	Number of nestling
86053	10.5.16	no	2	F	yes	12	18.3	65.5	0.551007	3	13	106	6	25	9	20	7	7
86056	10.5.16	no	1	F	yes	10.8	17.6	64.5	0.769303	3	NA	NA	NA	25	6	19	5	5
86058	10.5.16	no	2	F	yes	13	18.4	67	0.731321	3	NA	NA	NA	26	10	24	4	4
86062	11.5.16	no	1	F	yes	10.4	16.8	62	0.852451	3	24	89	6	25	11	20	3	3
86065	11.5.16	no	4	F	no	13.2	18.4	66	0.534733	3	NA	NA	NA	35	8	24	7	3
86068	12.5.16	no	2	F	no	11.9	18.6	66	0.858289	3	NA	NA	NA	28	10	21	9	9
86072	12.5.16	no	1	F	no	11.8	18.5	67	0.709688	3	NA	NA	NA	36	7	23	7	7
86074	12.5.16	no	1	F	yes	12	18	69	0.777677	3	NA	NA	NA	33	10	29	8	7
86076	12.5.16	no	3	F	no	12.5	19	68	0.640376	3	37	110	3	37	9	26	9	1
86079	13.5.16	no	1	F	no	12	18.9	69	0.843617	3	NA	NA	NA	23	6	20	6	6
86081	13.5.16	no	2	F	yes	11.5	18.7	67.5	0.889333	3	NA	NA	NA	22	8	15	8	0
86082	13.5.16	no	2	F	no	11	18.8	69	0.675601	3	20	106	10	21	7	14	8	7
86088	18.5.16	no	3	F	yes	11.6	18.6	67	0.726346	3	32	101	10	19	10	17	8	7
86092	18.5.16	no	1	F	no	12.4	18.8	66	0.815134	3	41	121	4	24	8	20	8	8
86094	24.5.16	no	2	F	no	11.2	18	68	0.934292	3	NA	NA	NA	27	4	19	4	4
86095	24.5.16	no	4	F	yes	10.8	18	64.5	0.793907	3	NA	NA	NA	19	11	14	4	4
86097	24.5.16	no	2	F	no	9.8	18.9	68	0.904963	3	NA	NA	NA	18	11	20	7	7
86100	25.5.16	no	3	F	no	10.6	19.4	66	0.561203	3	30	109	6	18	9	17	8	8
86104	27.5.16	no	2	F	no	11.3	18.9	67	0.804194	3	NA	NA	NA	27	12	19	6	6
86105	27.5.16	no	4	F	yes	10.8	18	65.5	0.709236	3	NA	NA	NA	25	11	19	6	4
93239	9.6.16	yes	0	F	NA	NA	NA	NA	0.780265	3	25	112	9	NA	NA	NA	NA	NA
86049	9.5.16	no	2	M	yes	11	19.9	70	0.923344	4	16	121	6	29	13	25	13	0
86050	9.5.16	no	1	M	no	11.2	18.7	66	1.081583	6	28	113	2	25	10	19	10	10
86051	9.5.16	no	2	M	no	11.9	19.7	71	0.953699	4	NA	NA	NA	34	11	23	6	6
86055	10.5.16	no	1	M	no	11.5	18.3	67	0.7732	10	21	88	6	24	7	23	6	6
86057	10.5.16	no	3	M	yes	11.9	20	71	0.447862	5	30	126	6	20	9	18	6	6
86059	11.5.16	no	2	M	no	11.9	20.2	70	0.557413	5	NA	NA	NA	20	9	NA	NA	NA
86060	11.5.16	no	2	M	no	10.3	19	69.5	0.601766	5	22	79	9	25	9	21	8	8
86061	11.5.16	no	6	M	no	11.8	20.2	71	0.83188	5	NA	NA	NA	19	13	19	6	6
86063	11.5.16	no	1	M	yes	10.8	18.9	68	0.794088	5	17	108	2	20	11	14	10	0
86064	11.5.16	no	1	M	no	11.9	20	70.5	0.662092	5	31	129	9	24	11	19	10	5
86066	11.5.16	no	1	M	yes	11.2	19.1	65.5	0.550107	2	43	112	4	37	8	25	8	7
86067	11.5.16	no	1	M	no	10.5	18.1	68	0.505703	5	NA	NA	NA	29	8	23	7	3
86069	11.5.16	no	6	M	no	11.8	19.6	71	0.778991	9	NA	NA	NA	33	9	23	9	9
86070	12.5.16	no	2	M	no	11.4	19.2	71	0.789441	5	13	126	7	31	11	24	11	6
86071	12.5.16	no	1	M	no	11	19.1	71	0.642774	9	20	112	3	36	7	23	7	7
86073	12.5.16	no	2	M	no	11	19.4	71	0.518775	5	18	129	7	36	9	25	9	9
86075	12.5.16	no	1	M	yes	NA	NA	NA	0.789801	2	46	123	5	33	10	29	8	7

Sample	Collection date	Was chick	Age at sampling (minimum)	Sex	Survived	Body mass (g)	Tarsus length (mm)	Wing length (mm)	Relative telomere length	Plate (PCR amplification)	Hatching date	Body mass (g) 15 days	Brood size	Laying date (1 = 1 April)	Clutch size	Hatching date (1 = 1 May)	Number hatched eggs	Number of nestling
86077	12.5.16	no	2	M	yes	11.2	19	71	0.462038	5	20	107	9	37	9	26	9	1
86078	13.5.16	no	2	M	yes	10	18.9	69	0.670857	9	14	109	8	19	10	18	9	9
86080	13.5.16	no	2	M	no	11.7	20.2	69	0.630148	5	18	132	7	22	8	15	8	0
86084	13.5.16	no	2	M	yes	11	20.9	70.5	1.081687	9	NA	NA	NA	26	10	19	10	0
86085	18.5.16	no	3	M	yes	11.2	18.9	70	0.924399	2	32	115	4	23	10	21	9	9
86086	18.5.16	no	1	M	yes	11.2	18.8	67	0.957833	2	35	123	6	22	10	17	9	0
86087	18.5.16	no	4	M	yes	11.5	18.5	71	0.709884	5	24	105	7	26	4	25	2	2
86089	18.5.16	no	2	M	yes	11.2	19	68	0.872981	8	22	103	7	19	10	17	8	7
86090	18.5.16	no	1	M	no	11.8	18.9	68	0.783789	5	31	118	9	22	10	19	7	7
86091	18.5.16	no	1	M	no	9.8	18.4	67.5	0.841704	5	37	115	4	22	11	18	10	4
86098	24.5.16	no	1	M	no	9.9	18.8	67	0.742383	5	23	108	5	18	11	17	9	7
86099	24.5.16	no	3	M	no	11.3	19.4	70	1.040323	5	31	121	1	25	11	20	3	3
86101	27.5.16	no	1	M	yes	10.6	17.9	66	0.742655	2	NA	NA	NA	23	10	24	5	4
86103	27.5.16	no	2	M	yes	10.2	18.8	67	0.840516	5	NA	NA	NA	24	9	18	5	2
86106	27.5.16	no	3	M	yes	10.2	17.3	68.5	0.746753	2	NA	NA	NA	25	11	19	6	4
93290	1.6.16	yes	0	M	NA	NA	NA	NA	0.851449	1	17	110	7	NA	NA	NA	NA	NA
93313	2.6.16	yes	0	M	NA	NA	NA	NA	1.613054	8	18	110	6	NA	NA	NA	NA	NA
93314	2.6.16	yes	0	M	NA	NA	NA	NA	0.667629	1	18	106	6	NA	NA	NA	NA	NA
93321	2.6.16	yes	0	M	NA	NA	NA	NA	1.795717	1	18	126	3	NA	NA	NA	NA	NA
93328	3.6.16	yes	0	M	NA	NA	NA	NA	0.533303	1	19	121	7	NA	NA	NA	NA	NA
93345	3.6.16	yes	0	M	NA	NA	NA	NA	0.810183	1	18	106	9	NA	NA	NA	NA	NA
93151	4.6.16	yes	0	M	NA	NA	NA	NA	0.902173	1	20	115	6	NA	NA	NA	NA	NA
93158	4.6.16	yes	0	M	NA	NA	NA	NA	0.639826	1	20	125	7	NA	NA	NA	NA	NA
93196	6.6.16	yes	0	M	NA	NA	NA	NA	0.620676	1	23	115	7	NA	NA	NA	NA	NA
91466	25.5.17	no	1	F	NA	11.3	19.3	67	0.70026	3	25	112	NA	31	8	21	8	0
91467	25.5.17	no	1	F	NA	12.3	18.9	65	0.582351	9	NA	NA	NA	31	12	27	11	11
91468	25.5.17	no	2	F	NA	13	19.4	66	0.83275	4	39	112	4	33	10	25	6	6
91472	26.5.17	no	5	F	NA	10.8	17.4	66	0.74784	3	NA	NA	NA	23	11	18	10	8
91473	26.5.17	no	4	F	NA	10.9	18.4	66	0.646406	3	NA	NA	NA	18	11	16	7	7
91476	26.5.17	no	1	F	NA	11	18.9	66	0.916276	9	NA	NA	NA	20	12	17	6	5
91477	26.5.17	no	3	F	NA	10.1	18.2	65	0.593555	4	NA	NA	NA	17	8	17	5	5
91932	4.6.17	no	1	F	NA	NA	NA	NA	1.079615	4	NA	NA	NA	33	9	25	8	8
91940	7.6.17	no	3	F	NA	NA	NA	NA	0.760572	9	NA	NA	NA	25	10	24	7	7
91483	8.6.17	no	4	F	NA	10.2	17.6	66	0.897874	9	NA	NA	NA	NA	NA	NA	NA	NA
91484	8.6.17	no	2	F	NA	10.9	19.1	67	0.801859	9	NA	NA	NA	36	8	26	8	2
91489	9.6.17	no	1	F	NA	11.5	19.2	64	0.972585	4	22	126	7	33	9	29	9	9
91490	9.6.17	no	3	F	NA	10.9	17.6	64.5	1.111009	4	NA	NA	NA	31	11	25	10	10

Sample	Collection date	Was chick	Age at sampling (minimum)	Sex	Survived	Body mass (g)	Tarsus length (mm)	Wing length (mm)	Relative telomere length	Plate (PCR amplification)	Hatching date	Body mass (g) 15 days	Brood size	Laying date (1 = 1 April)	Clutch size	Hatching date (1 = 1 May)	Number hatched eggs	Number of nestling
91491	9.6.17	no	3	F	NA	12.2	19.6	68	0.92406	4	NA	NA	NA	32	8	25	8	8
91493	9.6.17	no	1	F	NA	9.6	17.8	63	0.87155	4	NA	NA	NA	34	8	29	8	3
91942	10.6.17	no	4	F	NA	NA	NA	NA	1.942485	9	NA	NA	NA	31	9	27	7	5
91444	5.5.17	no	1	M	NA	10.5	18.4	66.5	0.963912	1	18	106	9	24	11	22	10	10
91445	5.5.17	no	1	M	NA	10.8	18.6	67.5	0.90004	1	18	110	6	29	10	NA	0	0
91446	5.5.17	no	1	M	NA	11.6	18.7	68	0.867241	8	17	110	7	34	10	28	10	9
91448	9.5.17	no	4	M	NA	10.8	19.3	71	0.733521	8	NA	NA	NA	22	9	17	9	9
91449	9.5.17	no	1	M	NA	10.2	17.4	65	1.459763	1	NA	NA	NA	30	12	26	11	1
91450	9.5.17	no	1	M	NA	10.3	17.9	68	0.740361	8	NA	NA	NA	NA	NA	NA	NA	NA
91451	9.5.17	no	3	M	NA	10.2	18.1	69	1.000000	1	NA	NA	NA	37	8	31	7	7
91452	9.5.17	no	1	M	NA	11.2	20.4	66	1.090732	1	NA	NA	NA	NA	NA	NA	NA	NA
91453	9.5.17	no	1	M	NA	11.9	20.6	68.5	0.745669	10	NA	NA	NA	NA	NA	NA	NA	NA
91456	12.5.17	no	1	M	NA	11.5	18.6	68	0.592237	1	20	125	7	25	10	22	7	7
91457	12.5.17	no	3	M	NA	10.8	18.6	69	0.693003	2	27	128	4	28	11	21	4	4
91458	12.5.17	no	3	M	NA	10.7	17.1	70	0.866845	2	NA	NA	NA	31	10	22	10	10
91459	12.5.17	no	1	M	NA	11	18.8	68	1.008271	1	20	115	6	33	8	26	8	7
91460	19.5.17	no	2	M	NA	11.5	18.8	68.5	0.755158	10	NA	NA	NA	25	8	19	8	8
91461	19.5.17	no	1	M	NA	10.8	18.6	68	1.061071	1	19	121	7	21	11	19	10	6
91463	19.5.17	no	2	M	NA	11.3	19.1	70	0.936332	2	NA	NA	NA	31	11	28	2	1
91464	19.5.17	no	NA	M	NA	11.7	19.6	71	1.064558	9	NA	NA	NA	NA	NA	NA	NA	NA
91465	25.5.17	no	2	M	NA	10.6	18.3	69	0.61427	2	NA	NA	NA	31	8	21	8	0
91469	25.5.17	no	1	M	NA	11.4	19.1	69.5	0.643183	10	NA	NA	NA	33	10	25	6	6
91470	25.5.17	no	1	M	NA	10.9	19.3	68.5	0.648292	2	19	129	8	24	10	21	10	10
91474	26.5.17	no	4	M	NA	10.3	16.9	69	0.832675	2	NA	NA	NA	23	11	18	10	8
91479	26.5.17	no	1	M	NA	10.7	19.6	68	0.765468	1	23	115	7	20	12	17	6	5
91480	26.5.17	no	3	M	NA	10.2	18	68	0.819598	10	NA	NA	NA	17	8	17	5	5
91481	26.5.17	no	2	M	NA	11.4	19.2	69	0.785038	2	46	123	5	31	12	27	11	11
91482	26.5.17	no	1	M	NA	9.9	18.4	67	1.258217	1	18	106	6	27	12	21	9	8
91930	4.6.17	no	1	M	NA	NA	NA	NA	1.244093	4	23	121	7	20	4	19	3	2
91931	4.6.17	no	1	M	NA	NA	NA	NA	0.847193	4	20	122	7	33	9	25	8	8
91938	7.6.17	no	2	M	NA	NA	NA	NA	0.961453	4	NA	NA	NA	25	8	19	7	7
91939	7.6.17	no	4	M	NA	NA	NA	NA	1.055526	4	NA	NA	NA	25	10	24	7	7
93321	8.6.17	no	1	M	NA	11.8	19.5	68	1.353462	1	18	126	3	31	NA	NA	0	0
91941	10.6.17	no	2	M	NA	NA	NA	NA	0.97951	4	20	97	3	31	9	27	7	5