Telomere dynamics during nestling development in bluethroats (*Luscinia s. svecica*)

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- VIVIAN C. SØRHEIM

Abstract

Telomeres, the tandemly repeated sequences at the ends of vertebrate chromosomes, shortens during an organism's life due to internal and external factors. The conditions experienced by an individual during its growth period, can have a significant influence on fitness later in life. In this thesis I will describe the patterns of telomere change in the nestling period of bluethroats (*Luscinia svecica svecica*). Based on findings from an earlier study by Johnsen *et al.* (2017), I tested the hypothesis that the degree of early change in telomere length reflects individual differences in body mass and/or genetic quality of the birds. I predicted that the reduction of telomere length can be detected at an individual level and that it is correlated with their individual change in body mass. I also investigated the relationship between relative telomere length and various life-history, environmental and parental variables, using longitudinal data; samples from the same nestlings on two occasions, four days apart. Field work was conducted at the Natural History Museum's field station in Øvre Heimdalen, Innlandet. Blood samples from 64 individuals were assessed and the relative telomere lengths were estimated using qPCR analyses. My findings showed no significant change in relative telomere length between the two sampling days, and there was no significant correlation between the sampling days. The results indicated a significant association between relative telomere length and parents' tarsus length, as well as an effect of the qPCR plate they were analysed on. None of the other variables that were tested showed any significant relationships with relative telomere length. I discuss possible reasons for the discrepancies between this and previous studies.

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1 Introduction

Telomere dynamics, the change of an organisms telomere length over time, have been intensively studied since the discovery of telomeres in the late 1970s (Blackburn *et al.*, 1978), and is still heavily studied today. Telomere length shortens throughout an organism's life, and this attrition happens at higher rates during early development (Zeichner *et al.*, 1999). In humans, telomeres are related to cancer development (Shay *et al.*, 1996) and mortality (Cawthon *et al.*, 2003; Willeit *et al.*, 2010; Ridout *et al.*, 2018), but telomeres have also been shown to play vital roles for senescence of cells (Sozou *et al.*, 2001; Aubert *et al.*, 2008), growth (Hall *et al.*, 2004), life expectancy (Pauliny *et al.*, 2006; Bize *et al.*, 2009; Heidinger *et al.*, 2012), and reproductive success (Sudyka *et al.*, 2019). Telomeres have been thoroughly studied in many vertebrate species (e.g. Richter *et al.*, 2007; Nanda *et al.*, 2009; Plot *et al.*, 2012; Näslund *et al.*, 2015; Seeker *et al.*, 2018), including birds (Pauliny *et al.*, 2006; Vedder *et al.*, 2018; Viblanc *et al.*, 2020).

Eukaryotes have their genetic material arranged into linear chromosomes contained within the nucleus of their cells. At both ends of each chromosome there are specialized G-rich structures called telomeres, repeated nucleotide sequences, whose primary function is to work as a protective cap and provide stability to the chromosome ends, as well as to prevent chromosome degradation and fusion (Blackburn, 1991). These tandemly repeated DNA sequences are thousands of base pairs long and, in vertebrates, consists of $(TTAGGG)_n$ sequences which shorten with each cell division (Meyne *et al.*, 1989). This is due to a known problem at the ends of linear chromosomes which is commonly referred to as "the end-replication problem". During replication of a new lagging strand the standard DNA polymerase cannot completely copy its ends (Watson, 1972), eventually leading to shortening of the telomeres until a critical point of replicative senescence or programmed apoptosis (Olovnikov, 1996; Hornsby, 2002; Campisi, 2003). Telomerase, a ribonucleoprotein-carrying enzyme (Greider *et al.*, 1989), was identified as being able to elongate telomere 3′ ends by adding TTAGGG repeats, and seemed to be the solution to the end-replication problem (Greider *et al.*, 1985). However, the activity of telomerase alone in most cell lines is usually not high enough to prevent telomere attrition (Lansdorp, 2005), and even when it seems to be essential for maintenance of telomeres, it is inactive in most somatic tissue (Kim *et al.*, 1994). This inactivity might be a vital mechanism to

prevent tumour growth, as studies have shown that most malignant cancer cells in humans have re-activated or over-expressed telomerase (Kim *et al.*, 1994; Shay *et al.*, 1996). In birds, on the other hand, it has been theorized that small body-size, and thus smaller amount of cells, allows higher telomerase activity without the increased risk of cancer (Monaghan, 2010).

Although cell proliferation is the most known cause of telomere shortening, it is not the only one, as different studies have later shown several environmental stress-factors that also influence telomere shortening and accelerate the process of attrition. A common factor is oxidative stress, which can result in telomeric double-stranded breaks in the GGG-containing telomere structure (Henle *et al.*, 1999), and can be even more damaging than the end-replication problem itself (von Zglinicki, 2002). The amount of telomere damage from oxidative stress, that goes unrepaired to the next cell division, will influence telomere loss at that division (von Zglinicki, 2002). Oxidative stress has also proven to increase the rate of telomere attrition, both *in vitro* (sheep *Ovis aries* and humans *Homo sapiens*) (Richter *et al.*, 2007) and *in vivo* (mice, *Mus musculus castaneus*) (Cattan *et al.*, 2008). Other environmental factors that might increase the oxidative stress that an individual experiences, like alkylation (Petersen *et al.*, 1998) and UV-irradiation (Oikawa *et al.*, 2001), have also been shown to cause significant damage to telomeres.

The same pattern of telomeric change as for other vertebrates can be found in birds. Their telomeres shortens during their lifetime, although differently for shorter (Sudyka *et al.*, 2016) and longer-lived species (Haussmann *et al.*, 2003). This decrease is influenced by different external stress-factors as well as internal genetics (Monaghan *et al.*, 2006). The early-life stage is vital for telomere dynamics (Salomons *et al.*, 2009; Pauliny *et al.*, 2012), and the environmental conditions experienced can have important effects on survival later in life. The nestlings' body mass can influence telomere length (Hall *et al.*, 2004), but as growth rate is higher in the nestling period, the relationship seems to become negative with age (Barrett *et al.*, 2013). Brood size might indirectly impact the rate of telomere attrition in the nestlings due to stress, as a larger brood seems to increase a nestlings' baseline level of stress-hormones (Quirici *et al.*, 2016). Male nestlings tends to have longer telomeres than their female counterparts (Foote *et al.*, 2011) due to more germ line cell divisions (Zeichner *et al.*, 1999), which indicates that there might be variation in individual telomere length within the same age group. As an individual's telomeres are determined by its number of telomeric repeats (Blackburn, 1991), the initial telomere length

of an individual will in theory impact their future development of telomeres, as nestlings born with longer telomeres have more telomeric repeats to lose before they become critically short. However, a study on white-browed sparrow-weavers (*Plocepasser mahali*) suggest that it is the attrition of telomeres that largely predicts the reduced survival of the bird, and not the length of the telomeres itself (Wood *et al.*, 2019). The same study found longitudinal data from within-individual sampling to be more useful than cross-sectional data, as the repeated samples makes it possible to make precise calculations of the changes in telomere length. This suggests that long telomeres might not accurately predict the lifespan as earlier assumed (Heidinger *et al.*, 2012). The telomere length of nestlings might also be associated with the fitness and life-history traits of both the biological and social parents (Viblanc *et al.*, 2020). Studies suggests that the age of the male that sired the offspring might have an impact on telomere length reduction for the offspring (Parolini *et al.*, 2015; Noguera *et al.*, 2018). However, heritability seems to be higher between the female and her nestlings, than between the male and his offspring, as her telomeres, both at the time of reproduction and in early-life, is correlated to the telomeres of her nestlings (Asghar *et al.*, 2015; Johnsen *et al.*, 2017). Other studies found that nestlings reared under unfavourable conditions get shorter telomeres (Boonekamp *et al.*, 2014; Watson *et al.*, 2015). A study by Horváthová *et al.* (2012) on blue tits (*Cyanistes caeruleus*) found that in birds with bi-parental care, the female usually lay more eggs when mating with a high-quality male with longer telomeres. This might suggest that pairs that have a higher amount of eggs also will have nestlings with longer telomeres, and that it might indirectly reflect conditions and/or genetic quality of the female. However, a study by Noguera *et al.* (2016) found no effect of clutch size on embryonic telomere length.

In this project, I will describe the pattern of telomere change in the nestling period of bluethroats (*Luscinia s. svecica*). It is a small migrating passerine bird in the Muscicapidae family with a northern breeding distribution. The population in Øvre Heimdalen, Norway, has been intensively studied during the last 30 years (e.g. Johnsen *et al.*, 1995; Rekdal *et al.*, 2019) and is still monitored yearly, although not much research has been done on telomere dynamics in nestlings in this species. A recent cross-sectional study by Johnsen *et al.* (2017) found a negative correlation between nestling mass, reflecting age, and telomere length in bluethroat nestlings. I will build on that study and test whether the reduction in relative telomere length is a result of individual changes or e.g. differential mortality, using longitudinal data. I will also test whether

within-individual relative telomere change can be explained by a number of life-history and environmental factors, as well as parental traits. This will be done with a two-day sampling scheme of nestlings, four days apart, which makes it possible to also test if the degree of change in early telomere length reflects individual change in body mass and of the birds.

2 Materials and Methods

2.1 Study area and species

The fieldwork was carried out at Øvre Heimdalen field station (61°25'N, 8°52'E), located in Innlandet, Norway. It was conducted between the end of May and the beginning of July 2018. The valley is located at approximately 1100 meters above sea-level and the habitat is mainly above the tree line, except for the south facing valley-side that is dominated by subalpine birch forest.

The bluethroat is a small passerine bird that weighs approximately 18 grams. The male is slightly larger and very colourful compared to the female. It is socially monogamous (Cramp, 1988), but genetically promiscuous (Krokene *et al.*, 1996; Johnsen *et al.*, 2003). They are insectivorous and nests mainly on the ground, hidden under dwarf birches and other smaller shrubs. The female lays 5 – 7 eggs that is incubated for 13 – 15 days (Johnsen *et al.*, 1995), before the nestlings are fed by both parents.

The bluethroats in Øvre Heimdalen has been thoroughly studied, and studies on the population have revolved around extra-pair mating and female promiscuity, as well as plumage colouration of males, sexual selection and mate guarding (Fossøy *et al.*, 2007; Johnsen *et al.*, 1995). Therefore, as the protocols for the fieldwork were already well established, and there is a good amount of data collected of the species from Heimdalen, the bluethroat is an ideal model species for this master project.

2.2 Collection of data and field methods

Since this master thesis is a continuation of Johnsen and co-workers' project on telomeres in bluethroats from 2017 (Johnsen *et al.*, 2017), much of the field- and laboratory work will be based upon this work. The adult bluethroats were caught and sampled in two non-continuous periods in 2018, first between May 29th and June 6th, and then from June 19th to June 25th. Males were mostly caught in the first period, while the females were caught in the second period (the nestling period).

Adult birds were caught in their territories using mist nets and playback recordings from both sexes. A syringe needle and a capillary tube was used to draw 25 µl of blood from the brachial vein of each bird. Morphological measurements were collected, using a Pesola 50g spring balance to record mass (to the nearest 0.1 g), a wing ruler to measure wing length (to the nearest 0.5 mm), and a slide calliper to measure tarsus (to the nearest 0.1 mm). Birds were marked with a unique combination of one aluminium ring and three colour rings for later field identification at nests. Lastly, a photograph was taken of each bird with its plumage coloration and ring combination for later documentation. The sampling lasted no longer than 15 minutes for each bird, and they were released close to the capture site. Only the morphological measurements of adults will be used in this project, while the blood samples will be used by the Natural History Museum for other work.

Nests were located by observing the adults (especially the females during nest building) and their location marked on a map as well as in a Global Positioning System (Garmin) to easily locate it. Initially, 30 nests were monitored, but eight of the nests where then later lost to either predation or desertion. Four of the nests only had one sampling event (see below) and were not analysed. The females incubated the eggs between June 6th and June 15th, and nest visitation was therefore decreased during this period until the estimated date of hatching.

On day 2 and 6 after the first nestling had hatched (day 0), ~15 µl of blood was taken (brachial venipuncture) from each individual nestling using a syringe needle and a capillary tube. If a nest was found after it had hatched, the nestlings were sampled at once, and then again four days later unless the nestlings were estimated to be more than seven days at first sampling, in which case they were not resampled to avoid premature fledging. This resulted in some of the nestlings

being sampled on day 3 and 7 (4 nests), day 5 and 9 (1 nest), and 6 and 10 (2 nests) post-hatch, but always with 4 days between each sampling. Nine nests were sampled on day 2 and day 6. The approximate age of the nestlings was estimated using a growth curve from Rangbru (1994). Though the late sampling days were not ideal, the initial plan was in fact to do the second nestling sampling on day 7. This was changed to day 6 to get the latest possible sampling while still avoiding predation of the nestlings, as the risk of nestlings being predated were shown to be higher at a later time (Johnsen *et al.*, 2017). Mass was also measured from all the nestlings on both days, using a Pesola 10g spring balance (to the nearest 0.1 g).

The blood samples for both the nestlings and adults were collected and stored in 2 ml microtubes, containing 96% ethanol. After collection, all samples were stored in a refrigerator (4°C) at the Natural History Museum, University of Oslo, until DNA extraction. After extraction of DNA, the samples were stored in a fridge at 4°C until further analysis.

2.3 Ethical note

During the incubation period, females were avoided or released immediately if captured, to avoid nest desertion. The nests found in the building/laying phase were not visited between clutch completion and until the last few days of the incubation period, to reduce disturbance. Only a small amount of blood was taken from both adults (<25 µl) and nestlings (<15µl), and there was no sign of sampling or handling affecting their immediate survival, or fledging success of nestlings (personal observation). Authorization to collect blood samples from both adults and nestlings was given by the Norwegian Food Safety Authority (Mattilsynet). Authorization for ringing and mist net catching was given by the Norwegian Environment Agency (Miljødirektoratet).

2.4 Laboratory work

2.4.1 DNA Extraction with two different kits

Extraction of DNA from the blood samples was done using both the E.Z.N.A.® Blood and Tissue DNA Kit and the E-Z 96® Tissue DNA Kit (both Omega bio-tek). Both extraction sets were conducted according to the protocols of the manufacturer with the following changes (underlined):

E.Z.N.A ® Blood and Body Fluid Protocol

50 µl of the sample was mixed with 150 µl of elution buffer, bringing the volume up to 200 µl. 20 µl reconstituted OB Protease (Proteinase K) was then added and everything was vortexed before the mixture was incubated at 65°C for 30 minutes, with an occasional vortex during incubation to ensure cell lysis. Then 260 µl of absolute ethanol (RT, 96-100%) was added, before the entire lysate was transferred to a HiBind® DNA Mini Column. The column was centrifuged to bind DNA and the flow-through was discarded. After washing, the column was placed in a sterile 1.5 ml microfuge tube where 100 µl preheated (65°C) elution buffer (10mM Tris-HCl) was added, before the column was incubated at 65°C for 5 min. To elute DNA from the HiBind DNA Mini Column it was centrifuged, and the flow-through containing the DNA was retained. Lastly, a second elution step was performed as before, bringing the final volume of extracted DNA to 200 µl.

E-Z 96® Tissue DNA kit protocol – Blood

250 µl 0.01M diluted Tris-HCl was pipetted (using a multi-pipette) to blood in a deep-well plate, breaking apart the cell wall to more easily "free" the DNA. 250 µl of the whole blood and Tris-HCl mixture was transferred from each of the 96 wells in the deep-well plate, to each of the corresponding wells in a lysis plate. The plate was centrifuged briefly at 3700 rpm for 2 minutes to collect any solution from the mat. Every centrifuging from now on was done at 3700rpm. The plate was incubated at 60°C for 60 minutes in an incubator and centrifuged briefly. An E-Z 96 DNA plate was placed on top of a 96-well square-well plate, before the lysate was transferred

from the lysis plate to the E-Z 96 DNA plate and centrifuged for 13 minutes. 500 µl HBC buffer (diluted with 100% isopropanol) was added to each well, before the plate was centrifuged for 5 minutes. The filtrate from the centrifuging was discarded, and the 96-well square-well plate was reused. After washing, the plate was centrifuged for 7 minutes, the filtrate was discarded, and the 96-well square-well plate was reused. A second DNA Wash step was performed before centrifuging the empty plate for 25 minutes (this step was critical, as it removed the trace residual ethanol that might interfere with downstream applications). The filtrate and the 96-well square-well plate were discarded. The E-Z 96 DNA plate was transferred to the 96-well racked microtubes, and 200 µl elution buffer (heated to 70°C) was added to the wells before the plate was incubated at 70°C for 5 minutes and centrifuging it for 7 minutes. This made a total volume of 200 µl extracted DNA for each well, and the 96-well racked microtubes were sealed with the appropriate caps.

Concentrations of the DNA extracts were measured using the Invitrogen™ Qubit® 2.0 Fluorometer (ThermoFisher Scientific), using the Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA). The concentrations from both the kits ranged from 1,08 ng/µl to 48,3 ng/µl. Five samples that had a concentration of <0.01 ng/µl after extraction were assumed to be 0.25 ng/µl before they were run in the qPCR machine. This was only done on the last plates, as the samples that showed 0.01 ng/µl before I had this knowledge were excluded in the analysis. The dataset might have been bigger if this had been done consistently. The blood samples were stored in a refrigerator at 4°C before and during the DNA extraction, and so did the DNA extracts after extraction, as well as during the following qPCR-period of 11 months.

2.4.2 Estimating the T/S-ratio using a qPCR method

To estimate the T/S-ratio (referred to as relative telomere length from now on) of the samples, quantitative real-time Polymerase Chain Reaction (qPCR) was used. The same protocol used by Johnsen *et al.* (2017), was used, which was optimized for bluethroats by Angela Pauliny, University of Gothenburg, and based on the protocol by Cawthon (2002). The use of real-time qPCR enables monitoring of the amplification of PCR product in real time using SYBR® Green, a fluorescent double stranded DNA-binding dye. The idea behind this is that with each PCR

cycle the amount of product doubles and accumulate exponentially until there are no reaction components left to use, and the analysis has reached a stationary phase. The amount of amplified PCR product eventually reaches a threshold where the qPCR instrument may detect the emitted fluorescent signal, and the cycle for when this occur is referred to as the quantification cycle (Cq) (Cawthon, 2002; Criscuolo *et al.*, 2009). In this thesis, I used standard primers for telomeres, the single-copy reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Criscuolo *et al.*, 2009), and 20 ng of DNA from each sample per reaction. The control single copy gene GAPDH was amplified using the primers GAPDH-F (forward) and GAPDH-R (reverse), and the telomeres were amplified using the primers Tel1b (forward) and Tel2b (reverse) (Criscuolo *et al.*, 2009). These primers, initially specific for zebra finches, were verified and tested for bluethroats by Johnsen *et al.* (2017).

The first six plates and the last five were run with a two-month gap between them, as I was waiting for new qPCR plates to arrive at the laboratory. The qPCR was always performed in separate 96-well plates, one with the telomere primer mastermix and one with the GAPDH primer, and with their own qPCR program (programs in Appendix 1). This is because the GAPDH needs more cycles of PCR to produce equal amount of fluorescent signal to telomere (Cawthon, 2002). For both the standard curves and qPCR runs, the DNA samples had the same matching well-position on the telomere-plate as on the corresponding GAPDH-plate, to reduce the variability in the T/S ratio. A triplicate No Template Control (NTC), containing 4 µl of MilliQ H_2O instead of DNA, was included on all plates. This was to make sure that there was no contamination of the plates.

Protocol for the qPCR assessment

The concentrations of the DNA extracts were analysed using the Qubit® 2.0 on either the day of or the day before the qPCR analysis, depending on how early in the day it was. The qPCR analysis was conducted on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All samples were run on clear-well plates (BRAND® 96-well PCR plates, BRAND GmbH & Co KG). The first six plates were run with one type of clear adhesive seals (Microseal® 'B' Adhesive Seals, Bio-Rad), and the last five plates were run with seals (BRAND® Sealing Film for Microplates, BRAND GmbH & Co KG) from the same manufacturer as the wells. This

switch of seals reduced the overall evaporation of samples during the qPCR assay. All amplifications were run using the real-time qPCR reagent SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). An analysis of the produced melt curve was performed after each qPCR run to ensure the result of a specific PCR product. As the qPCR analysis is sensitive to differences in concentrations of both DNA and components in the SYBR® Green Supermix, extra care was taken to make sure the samples had equal amount of DNA by diluting them down to the same concentration (0.25 ng/µl), as well as making sure the pipetting technique did not vary between plates or wells. The same pipettes were used for the entirety of the qPCR assay, and only one person (me) did the pipetting. The pipetting of diluted sample and mastermix in the wells was always done in the same order; first the sample, then the mastermix. The reason for the mastermix being pipetted last was to get the least amount of degradation of the SYBR® Green Supermix due to heat and light exposure. The analyses of all samples were performed in a total of 11 separate plates, run over a six-month period, which was not ideal as the analysis of multiple plates should be accomplished in as short time as possible to maintain a consistent pipetting technique. This was not possible due to schedule constraints and an unforeseen two-month delay. The applicable Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009) were followed to ensure high quality, reliability and repeatability for all results.

I used a final concentration of 3.5 µM for the GAPDH-primers (forward and reverse), 2 µM for the Tel1b forward-primer and 4 µM for the Tel2b reverse-primer. These aliquots of diluted primers were used for analysis of the first six plates, and new aliquots with the same concentrations were made for the last five plates, as there was such a long time since the first ones were diluted.

Assessing amplification efficiency

Standard curves for telomeres and the GAPDH reference genes were run to examine the efficiency of the analysis (Svec *et al.*, 2015). The curves were run before the first qPCR analyses of samples, on separate plates from the amplified samples to ensure preciseness. A randomly chosen DNA sample was used as the basis to create a 45 µl stock with concentrations of 1 ng/µl and 2 ng/µl, for telomeres ad GAPDH, respectively. Then a serial dilution of 1:3 was made, with

the prepared stock, for six technical replicates each with six duplicates for each of the two concentrations (Appendix 2 for more information). A master mix was then prepared separately for each of the standard curves, with 5 µl 1X SYBR® Green Supermix and 0.5 µl of both forward and reverse primer. 4 µl of serially diluted DNA and 6 µl of master mix was pipetted into each well, making the total volume of 10 µl. The first plates were run in the middle of May 2019, and the last plate was run in the end of November the same year.

The qPCR software CFX Manager™ 3.1 (Bio-Rad) was used to generate the standard curves with R^2 and efficiency estimates. The R^2 represents the linearity of the experimental data, and yields a measure of variability across replicates of the same concentration as well as showing whether the amplification efficiency for different starting template copy numbers is the same (Taylor *et al.*, 2010). To be considered acceptable for further analysis, the optimal standard curve would ideally produce a linear standard curve (R^2>0.980) (Taylor *et al.*, 2010) and an amplification efficiency (E) between 85-115% (Criscuolo *et al.*, 2009) (calculated as $E = 10^{[-1/slope]}$ (Pfaffl, 2001), with little variation among triplicates of the same concentration. If the R^2 = 1 and the E-value = 100% it would mean a perfect doubling of product during each qPCR cycle (Svec *et al.*, 2015).

The telomere and GAPDH standard curves were run twice, the first about four months before, and the second two days before the first qPCR plates were run. The average efficiency of the telomere standard curve for the blood samples was 119.4%, R^2 = 0.930 (E% = 119.9% and R^2 = 0.902; E% = 118.9% and R^2 = 0.958), and for the GAPDH reference gene it was 113.4%, R^2 = 0.988 (E% = 115.1% and R^2 = 0.984; E% = 111.7% and R^2 = 0.992). For telomeres, the E exceeded the recommended range and R^2<0.980. Different pipettes were used on the two standard curves, with the last curve having the same pipettes used for the rest of the project.

Amplifying the samples

All samples of DNA extracts (1,08 ng/µl to 48,3 ng/µl) were diluted with purified Milli-Q H_2O to a 0.25 ng/µl working stock the day of, or evening before, qPCR amplification. The same working stock with DNA was used for amplification of both telomere and GAPDH. The assay took place on the same day but on separate 96-well plates, and with a difference in the qPCR

program between them, as the GAPDH needs more cycles of PCR to produce equal amount of fluorescent signal to telomere (Cawthon, 2002). The DNA samples had the same matching position on the T plate as on the G plate, to reduce the variability in the T/S ratio. A master mix was then separately prepared for each plate, containing 5 µl 1X SYBR® Green Supermix and 0.5 µl of each primer (forward and reverse, conc. in protocol). Each well contained 4 µl (1ng) of sample and 6 µl of master mix, making the total volume of the wells 10 µl.

Both samples from each individual nestling were run on the same plate to make repeated estimates of individual nestlings maximally comparable, and every DNA sample was amplified in triplicates. Each plate also included one triplicate NTC, to check for contamination, and three triplicate Inter Plate Calibrators (IPCs), to compare assays across all plates. When calculating the T/S-ratio, IPC1 was used as a reference sample, as this worked on all the plates. IPC1 is also used in the calculation of CV% for inter- plate. There was a total of 11 plates used in the analysis, some of them were run up to three times for the IPC to have a SD-value below the threshold (see below).

2.5 Data analysis

2.5.1 Using two different datasets

Field work were done in collaboration with several different people, including Arild Johnsen and

Sara Bergseth. The measuring of both the nestlings and adult birds were completed by several different people, and there might be inter-observer measuring differences because of this. All DNA extractions were done in collaboration with Sara Bergseth, while the laboratory work with the qPCR was done solely by me.

A total of 197 blood samples was run with the qPCR, making up 94 individual nestlings and 34 adults. Some of the samples analysed were removed due to lack of a second sampling post-hatch or if one of the sampling days did not yield sufficient DNA concentration. The Cq-value standard deviation (SD) threshold was set to 0.22, instead of 0.2 used by earlier students (Hol, 2018; Macneish, 2018), to increase the size of the main dataset. In the statistical analyses, I will focus on two different datasets, dataset 1 and dataset 2, which is a higher- and lower-quality dataset.

Dataset 1 consists of all nestlings with two sampling days, where both days had a SD of the triplicates <0.22 for both telomeres and the GAPDH reference gene, and where the IPC1 worked on the plate. For two samples, I removed one of the triplicate values since it was very different from the remaining two values, resulting in SD <0.22. The samples that did not meet the criteria for dataset 1, but still produced values in the qPCR run were assigned to dataset 2 (see Appendix 4 for more info on dataset criteria). In the end a total of 16 nests were used in the final analysis from both datasets. The only nestling in nest number 9 was sampled on eight consecutive days (from day 2 – day 9 post-hatch) and is visualised in a plot (Figure 4). Day 2 and day 6 of these samplings were included in dataset 1 as they met the criteria.

Finally, dataset 1 includes 36 samples of 18 individual nestlings, each with a sampling on two different days, coming from 11 nests. The additional dataset 2 consists of 92 samples from 46 individual nestlings with a two-day sampling, and 14 nests. Adult samples were removed from the final analysis due to a tight time schedule.

2.5.2 Interpreting the results and calculating the T/S-ratio with qPCR

The qPCR software used to generate the standard curves, CFX Manager™ 3.1 (Bio-Rad), was also used to collect and analyse data from the qPCR-analyses (see Appendix 3 for CFX Manager™ output). Each sample was run in triplicate, and a mean Cq-value was calculated. If the SD of the three Cq-values was >0.22, the sample was either re-run (up to three times) or excluded from the dataset. The intra-plate coefficient of variation (CV%) between samples, for dataset 1, was 4.19% for TEL (ranging between 2.17% and 9.45%, with n = 49 in total on all seven plates, including IPC runs) and 2.44% for GAPDH (ranging between 1.64% and 3.63%, n = 49). For dataset 2 the intra-plate CV% was 9.94% for TEL (2.88% - 21.48%, n = 100 in total on all 8 plates, including IPCs) and 3.46% for GAPDH (1.35% - 7.44%, n = 100). The inter-plate CV%, based on IPC1, on the 7 plates in dataset 1 was 5.26% for TEL and 3.16% for GAPDH. For the 8 plates in dataset 2, the CV% was 4.65% for TEL and 2.36% for GAPDH. The repeatability between the plates were not particularly good, and this could be a result of the long duration of the qPCR process. The NTCs never had a fluorescent signal that reached above the 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 and described for all samples using the following equation in Pfaffl (2001):

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

A factor E = (E%/100) + 1 was generated by using the amplification efficiency estimates from the telomere and GAPDH standard curves, calculated in CFX Manager™ 3.1. The ΔCq gives an estimate of how much each sample in the analysis differs from the reference DNA sample (IPC1). To obtain this, the mean Cq-value of each sample was subtracted from the mean Cq-value of the reference sample (IPC1 from each plate), resulting in a ΔCq_{target} (telomeres) and $\Delta Cq_{reference}$ (GAPDH). All cells should only contain one copy of the reference gene, and the T/S-ratio should correspond to their relative telomere length (Cawthon, 2002). All further statistical analyses are based upon this T/S-ratio.

2.5.3 Statistical analyses using R

All statistical analyses and graphical illustrations were generated using R 3.6.3. (R Core Team, 2017), and the packages LmerTest (Kuznetsova *et al.*, 2017), ggpubr (Kassambara, 2020), and Hmisc (Harrell Jr *et al.*, 2020), in RStudio. The data was inspected for normal distribution using Shapiro-Wilks tests, and a significance level of 0.05 was used as a threshold for dismissing the null hypotheses (H0). Visual inspection of histograms and QQ-plots were also used to evaluate the normality of the data. As I had two different datasets (dataset 1 and dataset 2), I did two different sets of tests. The main conclusions will be based upon the results from dataset 1, while dataset 2 acts as support of these conclusions.

The relative telomere length on the first and second day of sampling (referred to as “rTL1” and “rTL2”) and the change in relative telomere length (referred to as “rTL change”), showed normal

distribution in dataset 1 (p = 0.10, 0.052 and 0.23, respectively). This was supported by visual inspection of histogram and QQ-plot. Parametric tests (Students t-test, Linear Mixed Effects Regression (LMER) and Pearson correlation test) where thus used on this dataset. In dataset 2, only rTL2 showed normal distribution (p = 0.39), while rTL1 and rTL change did not (p = 0.0037 and p = 0.0025, respectively). rTL1 showed normal distribution after a square root transformation (p = 0.21), while rTL change could not be transformed as it included negative values. Therefore, as these variables were not normally distributed, non-parametric tests (Wilcoxon paired test and Spearman correlation) were used.

Nest ID was included in the LMER-test as a random factor to account for more than one sampled nestling in some nests, thus controlling for non-independent values. A set of independent variables was added (see Appendix 6 for all variables used) to inspect how much of the variation in relative telomere length that was related to these variables. rTL1, rTL2 and rTL change was used as response variables. As I found an effect of qPCR-plate on both rTL1 and rTL2 (see below), Plate ID was added to all models with rTL1 and rTL2 as response variables. rTL change did not show any effect of Plate ID, and therefore these LMER models were run without Plate ID as a control. To avoid over-parameterized models, each model from dataset 1 was run with only two predictor variables (including Plate), based on the convention of one variable per 10 data points (n = 18). Dataset 2 was run with four to five predictor variables (n = 46). To reduce the LMER models from dataset 2, a stepwise backwards elimination method, starting with the variable with the highest p-value, was used. The significance level for non-significant variables was estimated by adding them to the reduced model. The model assumptions were verified by checking residuals of the final models with visual inspection of QQ-plots, and all residuals were normal. As there was a high possibility for type I errors, due to the high number of tests, the Bonferroni correction method was used. The Bonferroni correction calculates a new significance level based on how many test that were conducted on a dataset (Whitlock *et al.*, 2015). Regression lines are added for visual purposes only in the figures illustrating non-significant relationships.

3 Results

3.1 Methodological effect of qPCR-plate

rTL2 was significantly affected by which plate the samples were on during qPCR (p = 0.011, t = -3.01, estimate ± SE = -0.13 ± 0.043). Plate ID had no statistically significant effects on rTL1 in the reduced model (p = 0.10, t = -1.83, estimate ± SE = -0.13 ± 0.071), however, in four out of five of the models with another variable there was a statistically significant to marginal effect of Plate ID on rTL1 (p = 0.05, 0.081, 0.074, 0.057). By plotting Plate ID and rTL for both days against each other, the length seems to get shorter for each qPCR-plate that was run (Figure 1). For dataset 2, Plate ID showed a marginally significant effect on rTL2 (p = 0.084, t = -1.90, estimate ± SE = -0.13 ± 0.07; Appendix 8, Figure 11), but not for rTL1 (p = 0.24, t = -1.24, estimate ± SE = -0.11 ± 0.093) or rTL change (p = 0.84, t = -0.21, estimate ± SE = -0.019 ± 0.09).

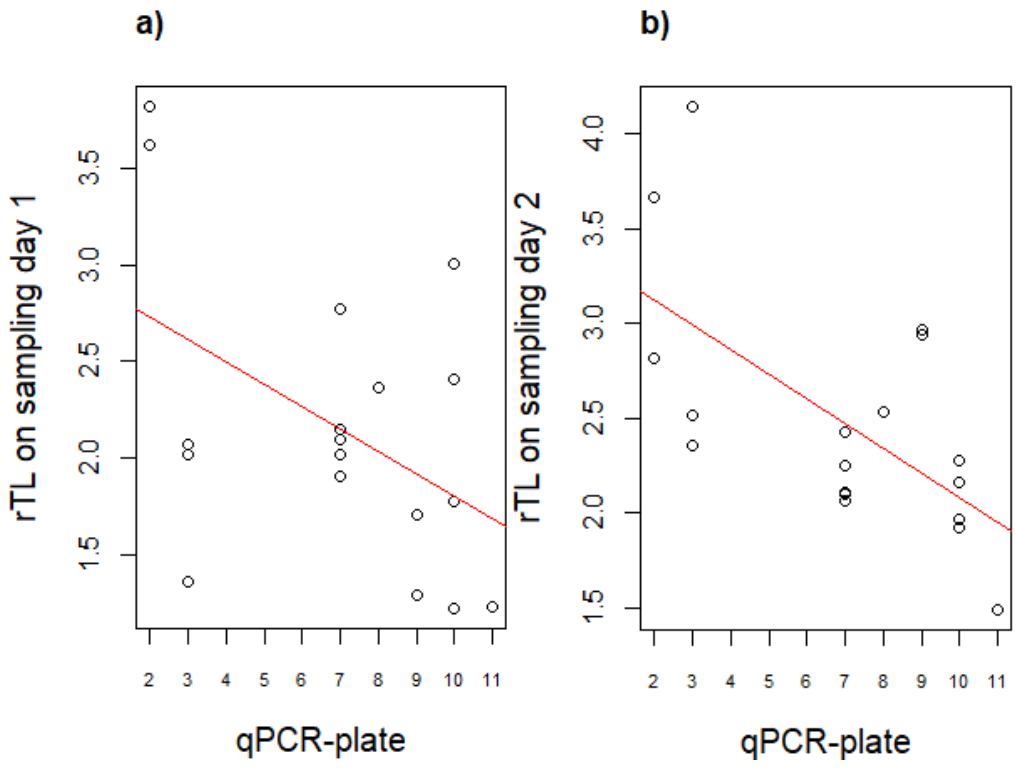


Figure 1. *Relative telomere length on a) sampling day 1, and b) sampling day 2, in relation to Plate ID for bluethroat nestlings. Dataset 1, n = 18.*

3.2 Change in relative telomere length between sampling days

Surprisingly, telomeres showed a slight overall increase (11%) from the sampling day 1 to sampling day 2. However, the increase was not statistically significant (Students' t-test, t = -1.79, df = 17, p = 0.091, n = 18; Figure 2). The additional dataset 2 supported these findings, with a 10.1% increase in telomere length from sampling day 1 to sampling day 2, with no statistical significance (Wilcoxon matched pairs signed rank test, V = 461, n = 46, p = 0.38; Appendix 8, Figure 12). Apparently, the two sampling days tended to be positively correlated (dataset 1, Figure 3; dataset 2, Appendix 8, Figure 13), although this correlation disappeared when Plate ID was controlled for. This was done by doing an LMER test with rTL2 as the response variable and rTL1 and Plate ID as predictors.

For the nestling in nest number 9, the plot shows a very varied development of rTL over time (Figure 4). From the plot it seems that the nestling's relative telomere length is getting longer and then shorter again before a new increase. As all the samples (except from the last one) of this nestling was run on the same qPCR-plate, the plate-effect cannot be an explanation to this unusual variation.

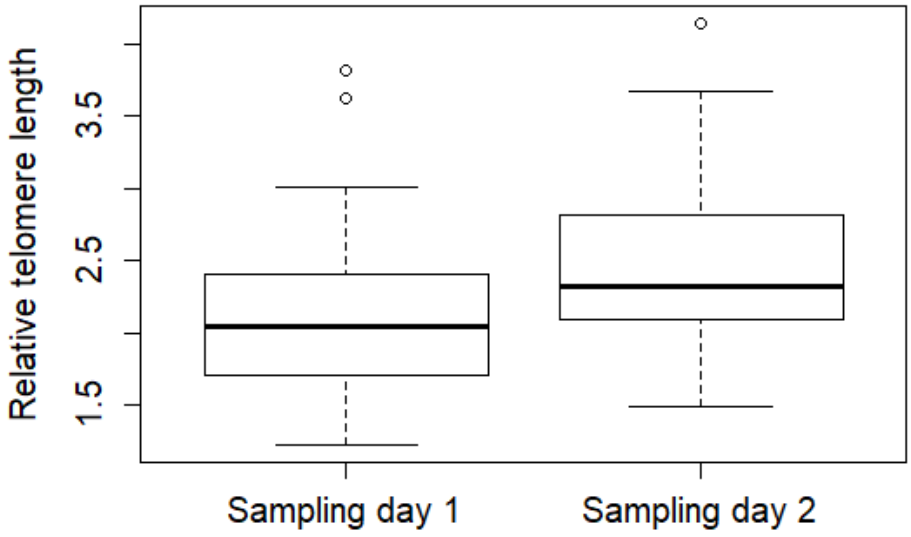


Figure 2. *Boxplot of difference in relative telomere length for bluethroat nestlings between sampling day 1 (rTL1) and sampling day 2 (rTL2). The black horizontal line shows the median (2.32 and 2.05), circles shows the outliers, and whiskers represent the lower (first) and upper (fourth) quartiles. Dataset 1, n = 18.*

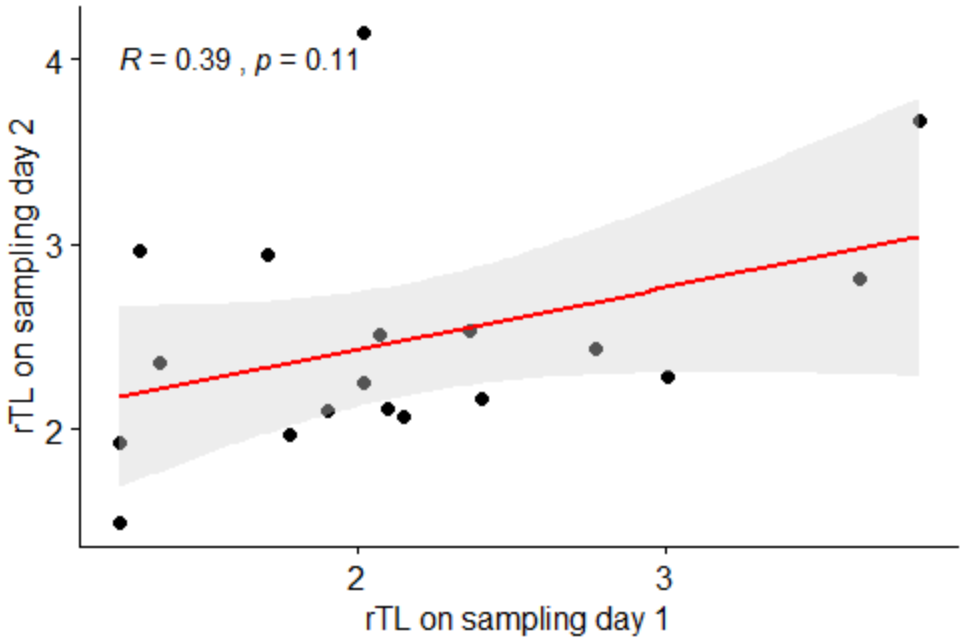


Figure 3. *Relative telomere length for bluethroat nestlings on the sampling day 2 in relation to sampling day 1. The grey area shows the 95% confidence interval. Dataset 1, n = 18.*

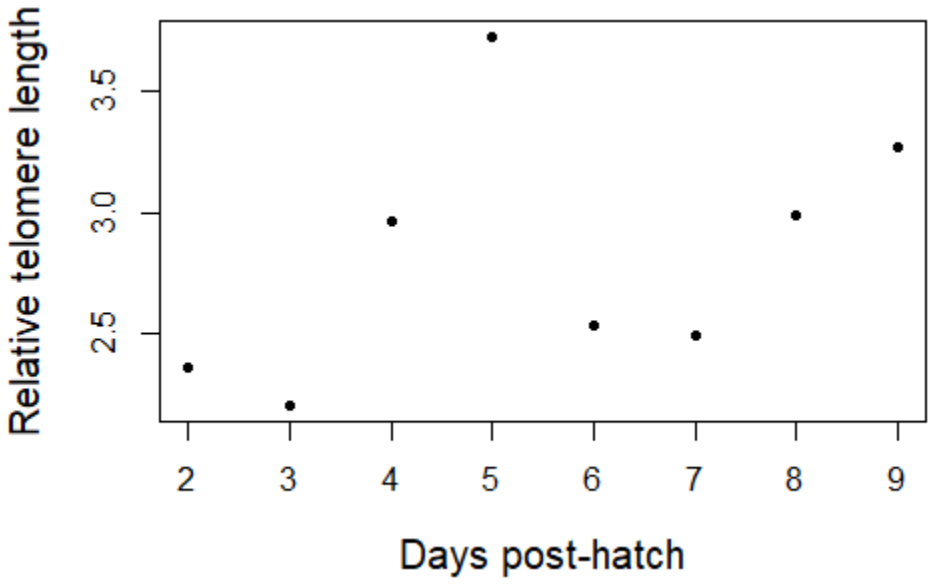


Figure 4. *Development of relative telomere length over time for the bluethroat nestling in nest number 9, n =1.*

3.3 Variation in relative telomere length in relation to nestling growth and environmental factors

There were no significant relationships between rTL for the two days and nestling mass on the day of sampling (Figure 5), nor between nestling change in body mass and change in rTL (Figure 6). rTL for nestlings on sampling day 1 and 2 were not correlated to neither the time nor date of sampling. Precipitation seemed to have a weak correlation with sampling day 1 ($p = 0.063$; Table 1; Figure 7), although not significantly so. Temperature was significantly correlated with rTL2 ($p = 0.0146$; Table 1; Figure 8). There was no correlation between brood size and rTL on either of the sampling days. None of the variables showed any significant relation to rTL change. Dataset 2 showed no significant relationships between any of the environmental factors (including body mass and change in body mass, Appendix 8, Figure 14 and 15) and rTL or rTL change (Appendix 7, Table 3).

Response variable	Variable	Pr(>|t|)	t	df	Estimate ± SE
rTL1	Date	0.17	1.52	7.69	0.20 ± 0.13
	Time	0.27	-1.2	7.78	-0.059 ± 0.049
	Mass	0.15	-1.52	13.9	-0.12 ± 0.079
	Precipitation	0.063	-2.16	7.96	-0.048 ± 0.022
	Temperature	0.62	-0.51	7.8	-0.22 ± 0.44
	Brood size	0.68	0.43	7.96	0.063 ± 0.15
rTL2	Date	0.55	0.62	8.47	0.053 ± 0.086
	Time	0.76	-0.32	9.64	-0.015 ± 0.046
	Mass	0.44	0.85	4.3	0.036 ± 0.042
	Precipitation	0.84	-0.21	8.79	-0.013 ± 0.065
	Temperature[a]	0.015	2.76	15	0.395 ± 0.143
	Brood size	0.52	0.66	11.1	0.056 ± 0.085
rTL change	Date	0.31	-1.08	10.3	-0.15 ± 0.14
	Time	0.16	1.55	8.75	0.065 ± 0.055
	Change in body mass	0.55	-0.61	13.9	-0.070 ± 0.11
	Precipitation	0.18	1.44	8.88	0.036 ± 0.025
	Temperature	0.71	-0.39	9.12	-0.15 ± 0.40
	Brood size	0.74	0.35	9.22	0.046 ± 0.13

Table 1. *Output from the LMER tests of all environmental factors for bluethroat nestlings, with nest ID as a random factor. Plate ID was included in all models for rTL1 and rTL2. Significant and marginally significant values are marked in yellow. Dataset 1, all n = 18.*

[a] *The model had an error message (isSingular) in R during testing, indicating too low variance.*

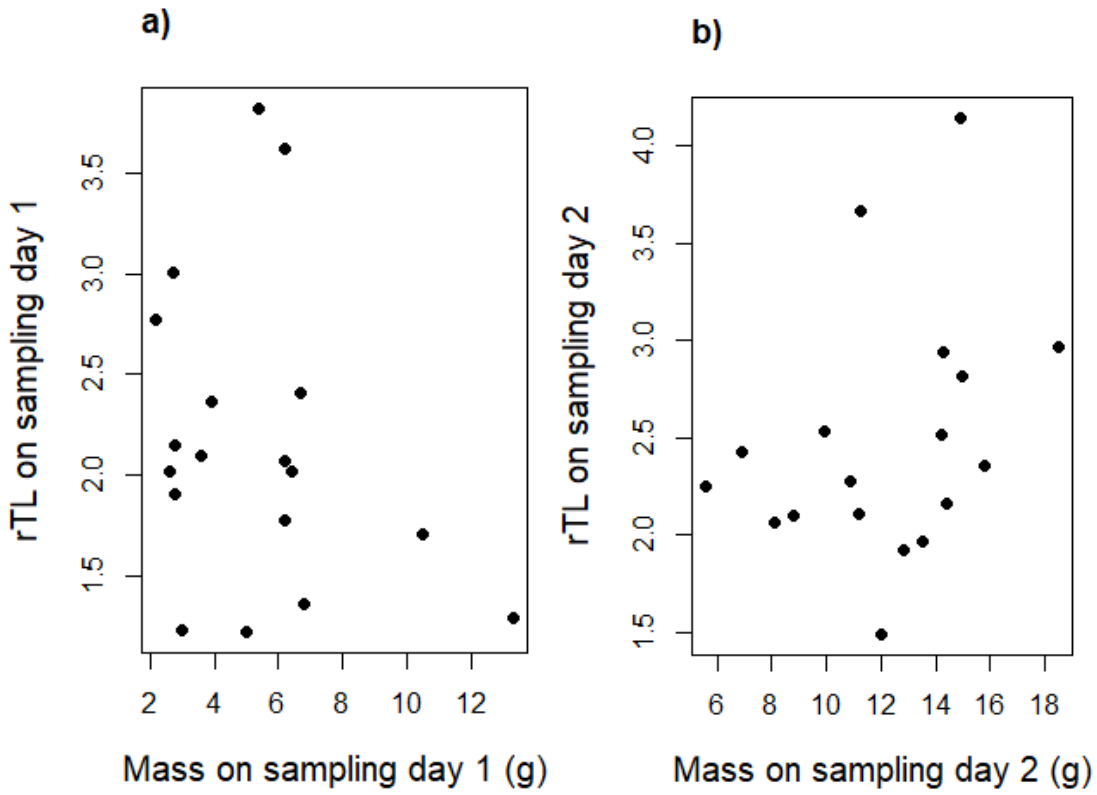


Figure 5. *Relative telomere length for bluethroat nestlings in relation to mass for a) sampling day 1, and b) sampling day 2. Dataset 1, n = 18.*

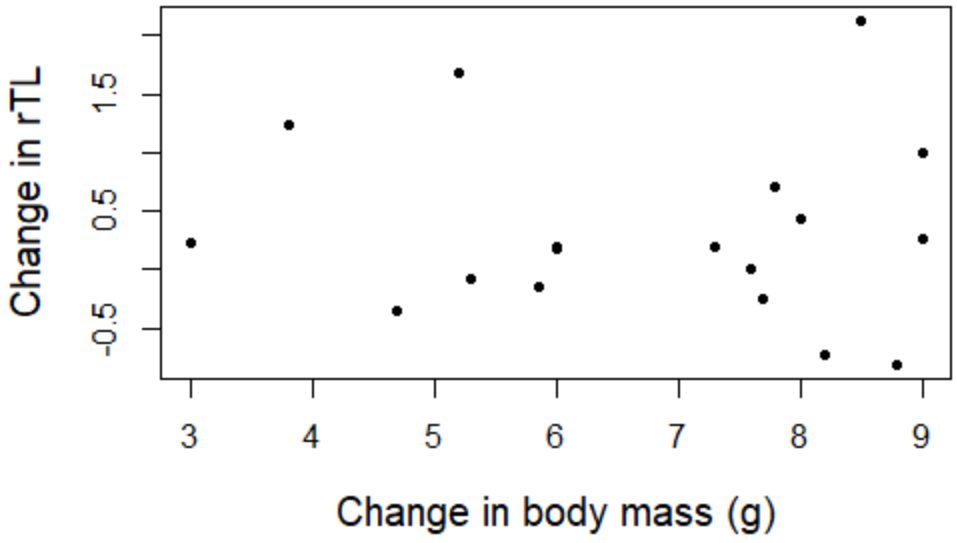


Figure 6. *Change in relative telomere length in relation to change in body mass (growth rate) for bluethroat nestlings. Dataset 1, n =18.*

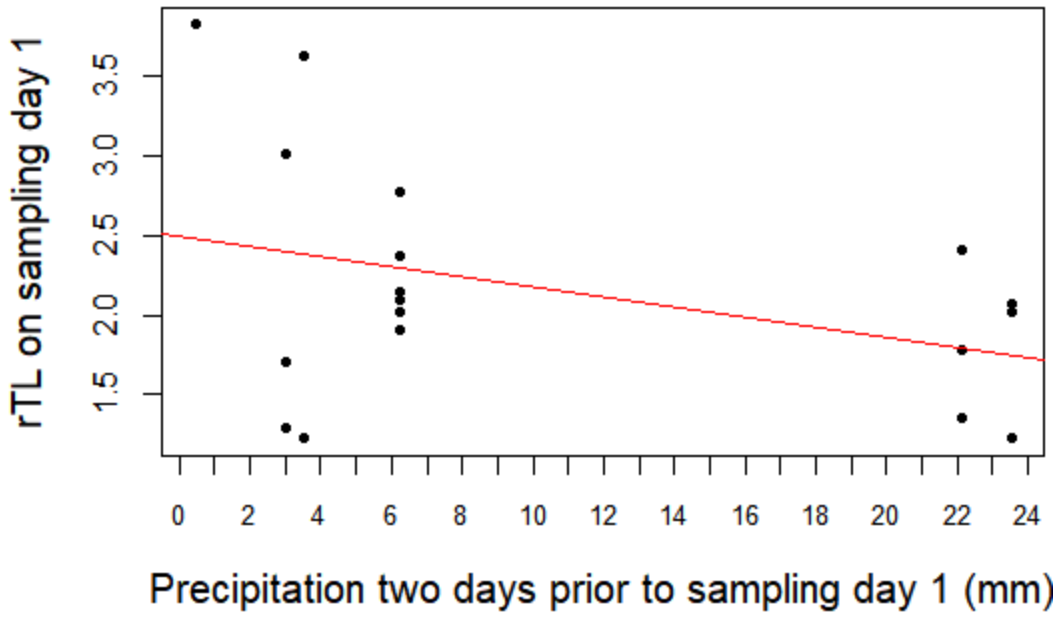


Figure 7. *Relative telomere length in relation to precipitation on the two days prior to sampling day 1, for bluethroat nestlings. Dataset 1, n =18.*

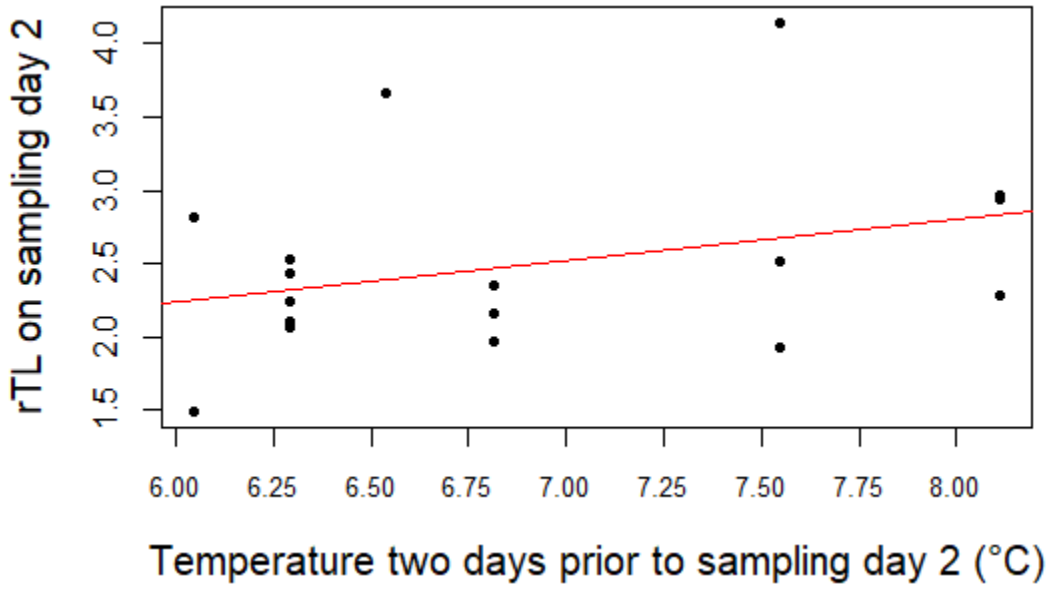


Figure 8*. Relative telomere length in relation to temperature on the two days prior to sampling day 2, for bluethroat nestlings. Dataset 1, n =18.*

3.4 Variation in relative telomere length in relation to parental factors

Female tarsus length showed a statistically significant, positive correlation with rTL1 (p = 0.006; Table 2, Figure 9a), and tended to be negatively correlated with rTL change (p = 0.07; Table 2, Figure 10), although not significantly so. rTL1 also had a statistically significant correlation with male tarsus length (p = 0.045; Table 2, Figure 9b). There was no correlation between any of the parental factors and rTL2, and rTL change was not related to any of the males' morphological traits. Clutch size showed no correlation with rTL on either of the sampling days, nor to rTL change.

For dataset 2, the same correlation between female tarsus length and rTL1 was observed (p = 0.032, t = 2.48, estimate ± SE = 0.41 ± 0.17, reduced model; Appendix 8, Figure 16a), as well as a negative correlation with female wing length (p = 0.033, t = -2.43, estimate ± SE = -0.085 ± 0.035, reduced model; Appendix 8, Figure 17) in the same model. There was also a significant relationship between rTL2 and female tarsus length (p = 0.026, t = 2.52, estimate ± SE = 1.03 ± 0.41, reduced model; Appendix 8, Figure 16b). Female tarsus length was not correlated with rTL change (Appendix 7, Table 4), however, female wing length showed a statistically significant negative correlation to rTL change (p = 0.013; Appendix 8, Figure 18). There were no relationships between clutch size or any of the males' morphological traits and rTL on either sampling day or rTL change in dataset 2.

Response variable	Variable	Pr(>|t|)	t	df	Estimate ± SE
rTL1	Clutch size	0.97	-0.04	7.88	-0.023 ± 0.59
	Female mass (g)	0.12	1.774	7.67	0.23 ± 0.13
	F tarsus length (mm)	0.006	3.535	9.32	1.19 ± 0.34
	F wing length (mm)	0.89	0.148	7.57	0.014 ± 0.094
	Male mass (g)	0.66	0.459	7.2	0.15 ± 0.32
	M tarsus length (mm)	0.045	2.386	7.76	0.71 ± 0.3
	M wing length (mm)	0.86	0.186	7.77	0.019 ± 0.1
rTL2	Clutch size	0.31	1.06	11.1	0.35 ± 0.33
	Female mass (g)	0.65	0.47	11.1	0.042 ± 0.091
	F tarsus length (mm)	0.4	0.87	12.2	0.28 ± 0.32
	F wing length (mm)	0.55	0.62	8.95	0.033 ± 0.053
	Male mass (g)	0.48	-0.747	7.28	-0.13 ± 0.17
	M tarsus length (mm)	0.66	0.455	7.06	0.098 ± 0.22
	M wing length (mm)	0.81	-0.243	10.02	-0.014 ± 0.06

rTL change	Clutch size	0.53	0.65	8.79	0.37 ± 0.57
	Female mass (g)	0.23	-1.29	8.95	-0.18 ± 0.14
	F tarsus length (mm)	0.07	-2.01	10.7	-0.91 ± 0.45
	F wing length (mm)	0.83	0.23	7.96	0.021 ± 0.092
	Male mass (g)	0.35	-1	7.5	-0.3 ± 0.3
	M tarsus length (mm)	0.13	-1.71	8.45	-0.57 ± 0.33
	M wing length (mm)	0.73	-0.36	8.63	-0.036 ± 0.098

Table 2. *Output from the LMER tests of all parental factors on bluethroat nestlings' telomeres, with nest ID as a random factor. Plate ID was included in all models for rTL1 and rTL2. Significant and marginal values are marked in yellow. Dataset 1, all n = 18.*

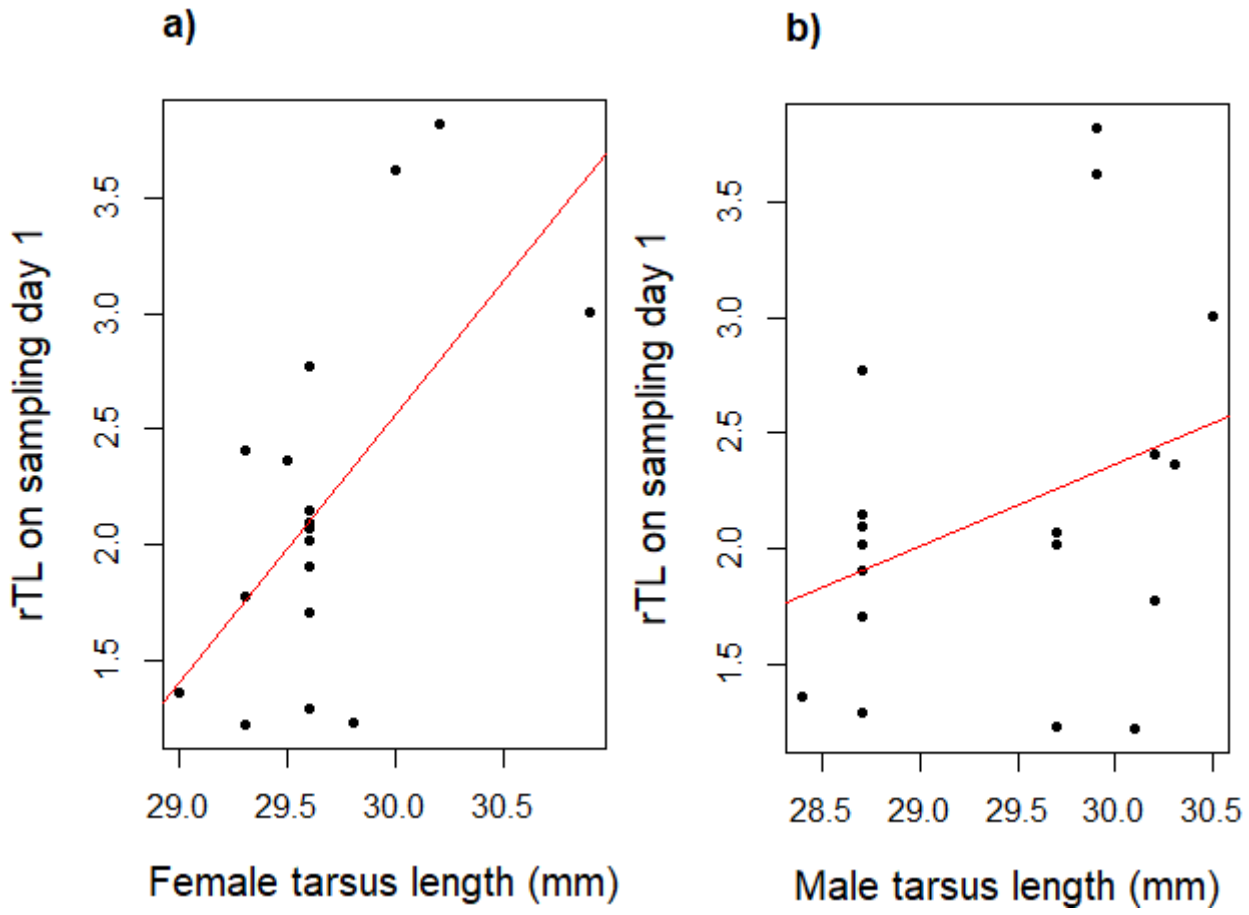


Figure 9. *Nestling relative telomere length on sampling day 1, in relation to male and female tarsus length, for bluethroats. Dataset 1, n = 18.*

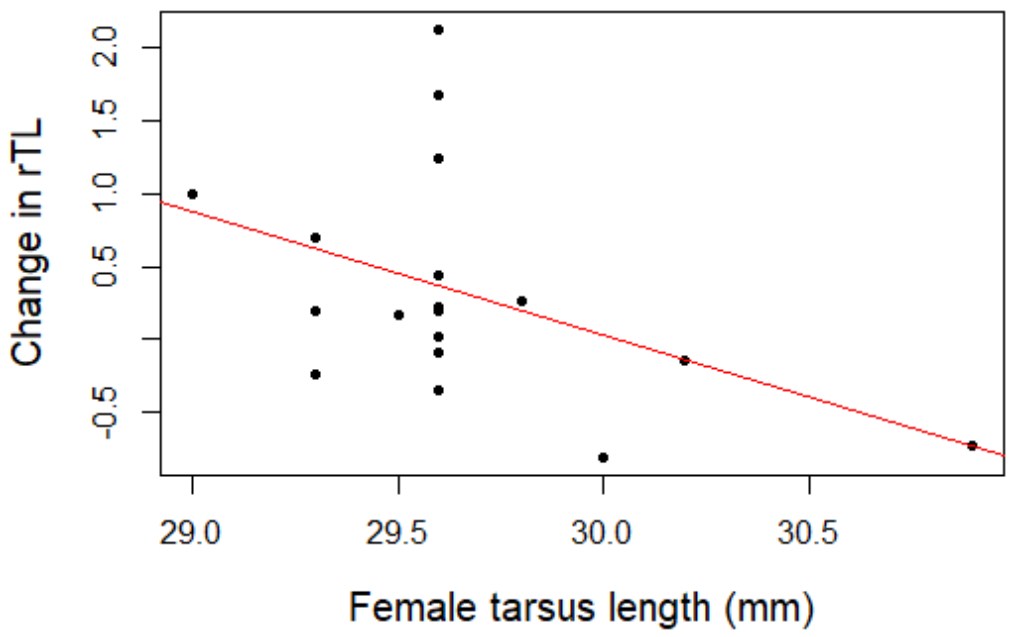


Figure 10. *Nestling change in relative telomere length in relation to female tarsus length, for bluethroats. Dataset 1, n = 18.*

4 Discussion

In this study, a longitudinal approach was used to describe the individual telomere development of bluethroat nestlings between two sampling days and their correlation to one another, as well as to analyse the nestlings' telomere length in relation to several environmental and parental factors. The results showed a slight overall increase (11%) in the relative telomere length from sampling day 1 to sampling day 2, although this was not significant. In dataset 1, ~67% of the nestlings showed an increase, while ~54% of the nestlings showed an increase in dataset 2. The lack of significance suggests that there is no systematic change in the relative telomere length, on an individual level, early in the nestling period of bluethroats. I found no correlation between the relative telomere length of the two sampling days in either of the datasets, when the plate-effect was controlled for. Furthermore, results did not show any significant correlation between mass and relative telomere length as found earlier, or between the change in relative telomere length and change in body mass. Of the environmental factors, precipitation and temperature seemed to have marginal relationships with relative telomere length on the first day of sampling (rTL1). Of

the parental factors, female tarsus length (rTL1 and rTL change) and male tarsus length (rTL1) was significantly correlated to the nestlings' telomere length. In dataset 2, female tarsus length (rTL1 and rTL2) and female wing length (rTL1 and rTL change) was significantly correlated to the nestlings' telomere length. These results were partly corroborated by dataset 2.

4.1 The telomere dynamics on an individual level

My findings suggest that the relative telomere length did not change significantly on an individual level. The results are different from the study by Johnsen *et al.* (2017), who found a clear reduction in telomere length during the first week after hatching in a cross-sectional study (samples collected in 1998 and 1999) of bluethroats and suggested it could be explained by a high number of cell proliferation and rapid growth in the nestling period. This has been hypothesized in other studies as well (Zeichner *et al.*, 1999; Ringsby *et al.*, 2015) and seems like a theoretically sound explanation. There are at least two possible explanations for this discrepancy. First, my dataset might have been affected by different factors than Johnsen *et al.* (2017) as the samples were collected in different years and on different birds. 2018 was an extraordinarily warm year, and food might have been more abundant during this breeding- and nestling season than in 1998/1999. This could mean that nestlings from 2018 were less exposed to cold-stress than those from 20 years ago, as well as not having to compete as rigorously for food. Both these factors could have led to less oxidative-stress in 2018, and therefore telomere attrition may have been less severe. Although I do not have the weather data for these years, this external factor cannot be ruled out as a possible explanation. Second, Johnsen *et al.* (2017) based their study on cross-sectional data rather than longitudinal data, which makes it harder to detect individual dynamics in telomere length. Differential mortality might also affect this, e.g. by surviving nestlings growing to become larger and tending to have shorter telomeres than the nestlings that did not survive, which would not have been sampled. If this is the case, their study might be a result of a biased selection, and the changes they found in their study might not reflect individual reduction in telomere length.

The results showed a non-significant increase (11%) rather than a decrease, which is contrary to other longitudinal studies (Boonekamp *et al.*, 2014; Viblanc *et al.*, 2020) showing a reduction

during the nestling phase. A study from Parolini *et al.* (2015) found a reduction, of approximately 10% telomere shortening, between 7 and 16 days of age in barn swallows (*Hirundo rustica*). However, the study also had an increase in relative telomere length in approximately 40% of their individuals. A possible reason for this is the variation in initial telomere length between individual nestlings. A general reduction of relative telomere length might have been discovered if my data had a larger interval between the samplings. This was not possible due to the high risk of predation after day 7, and the fact that bluethroats have a short nestling period of approximately 14 days. In a longitudinal analysis of European shag nestlings (*Phalacrocorax aristotelis*), Hall *et al.* (2004) reported a considerable amount of variation in telomere length for both individual nestlings and in telomere change, and suggested that this was a result of differences in the individual response to different telomere-modulating factors. The differences in chronological age could have an influence on the variation in the attrition of telomeres among nestlings, however, other studies suggests that telomere length reflects biological age rather than chronological age, as the variation was also found within the same age group (Bize *et al.*, 2009). Another possibility is that telomerase may be active in the earlier stages of the nestling period in bluethroats. Telomerase does not just occur in gametes, but also in the bone-marrow of bird nestlings (Haussmann *et al.*, 2004, 2007), and the study by Haussmann *et al.* (2004) found that in short-lived bird species, telomerase had high activity during the nestling period, before being down-regulated as the bird got older.

I found no relationship between nestling mass and relative telomere length, or between change in body mass and the change in relative telomere length, which is contrary to my predictions based on Johnsen *et al.* (2017), and to other studies (Hall *et al.*, 2004; Barrett *et al.*, 2013). In the study by Johnsen *et al.* (2017), mass was significantly correlated with relative telomere length of bluethroat nestlings sampled once from 2 – 13 days after hatching. Another study using longitudinal data of the lesser black-backed gull (*Larus fuscus*), also suggested that there were effects of growth on relative telomere length (Foote *et al.*, 2011). However, that study had more information on their individual samples, like sex of the nestlings as well as knowing the parentage of the different nests. They also found that males of their study species usually had longer telomeres at the point of hatching than their female counterparts (Foote *et al.*, 2011). Parolini *et al.* (2015) found differences in the dynamics of the sexes in the nestling period of barn swallows and suggested that the covariance of relative telomere length and growth depends on

both sex and sex-ratio within the brood. This might be because of the differences in size between the sexes, as males on average are larger than females and thus experience more cell divisions. Future studies should take such sex-differences into account.

The results showed a plate-effect on both rTL1 and rTL2, as the relative telomere length seemed to decrease with each progressing qPCR-plate. As there was approximately six months between the first and the last qPCR run, this might be due to degradation of the DNA extracts stored in the fridge. However, the effect of qPCR-plate might also be due to related nestlings being run on the same plate. Running nestlings from the same nest on different plates can control for this in future studies.

4.2 Environmental and life-history parameters

My findings showed that precipitation on the two days before sampling, was marginally correlated with relative telomere length on sampling day 1. I also found a correlation between temperature on the two days before sampling, and relative telomere length on sampling day 2. A possible biological interpretation of this might be that higher temperatures and lower precipitation lead to less oxidative stress for the nestlings. However, an alternative explanation is that these are statistical artefacts, resulting from the high number of tests, increasing the probability of getting false positives (type I error) (Whitlock *et al.*, 2015). None of these weather-parameters showed any statistically significant correlation with relative telomere on sampling day 1 after Bonferroni correction.

There was no correlation between relative telomere length, or the change of it, and brood size. This corroborates a study conducted by Voillemot *et al.* (2012), on collared flycatchers (*Ficedula albicollis*), that found no significant effects of brood size on telomere length, and suggested that telomere length at the end of the growth period does not accurately reflect the past developmental conditions after hatching. A difference to this study is that they measured nestlings later in the growth period. Another study, by Nettle *et al.* (2013), got similar results when analysing brood size, but found that the number of heavier competitors in the brood had an impact on telomere length, rather than just brood size. Most of the nests from my study had broods with six nestlings, and I did not perform the analysis of number of heavier competitors. A

larger brood will in theory increase the amount of oxidative stress a nestling is experiencing, and support for this theory is found in a recent cross-sectional study on nestlings of the Thorn-tailed Rayadito (*Aphrastura spinicauda*) that found a significant correlation between brood size, the baseline corticosterone levels and telomere length (Quirici *et al.*, 2016). However, as mentioned earlier, 2018 possibly had a large abundance of food, thus providing a possible explanation for the lack of correlation between brood size and relative telomere length.

4.3 Effects of paternal and maternal factors on relative telomere length

Clutch size could indirectly reflect the condition and/or genetic quality of the female, although I did not find a significant correlation between this maternal factor and the relative telomere length in her offspring. However, I did find a significant positive correlation between the females' tarsus length and the nestlings' relative telomere length and a negative correlation with her offspring's change in relative telomere length. This suggests that females with longer tarsi might produce offspring with initially longer telomeres that also tends to have a higher rate of telomere attrition than nestlings with initially shorter telomeres. Tarsus length is defined mostly by heritable factors, but environmental conditions may also be involved. There was also a correlation between male tarsus length, and his nestlings' relative telomere length, but as this was not supported by the nestlings change in relative telomere length or the additional dataset it might be a spurious significance. There is also the fact that bluethroats are a promiscuous specie (Johnsen *et al.*, 1995; Johnsen *et al.*, 1998), and the male that was feeding at the nest might not be the biological father of all the nestlings in the clutch. Thus, this result might not accurately reflect a relationship between nestling relative telomere length and paternal traits. To investigate this further, a paternity analysis of all the nestlings should be conducted to determine the parentage, and therefore get more accurate results.

5 Conclusion

This study is the first to investigate the individual change in relative telomere length over a two-day sampling of bluethroat nestlings. The degree of early change in telomere length does not reflect the individual nestlings' change in body mass. External environmental factors like precipitation and temperature might be associated with the telomere length on certain days of nestlings, but not the actual change in relative telomere length. The study also suggests a significant correlation between females' tarsus length and her nestlings' telomere length and the change of it. A larger dataset is to be preferred for future studies, as it yields more precise results. The bluethroat might not be the most optimal species to investigate the individual change during the nestling period, as it has a high level of predation and its nestling period is short. More research should be instigated to get clarity in the development of relative telomere length on an individual level, as well as the relationship between telomere length in early-life and environmental- and parental factors.

References

Asghar, M., Bensch, S., Tarka, M., Hansson, B., & Hasselquist, D. (2015) Maternal and genetic factors determine early life telomere length. *Proceedings of the Royal Society. B, Biological sciences*, **282**: 1-9.

Aubert, G., & Lansdorp, P.M. (2008) Telomeres and aging. *Physiological reviews*, **88**: 557.

Barrett, E.L.B., Burke, T.A., Hammers, M., Komdeur, J., & Richardson, D.S. (2013) Telomere length and dynamics predict mortality in a wild longitudinal study. *Molecular Ecology*, **22**: 249-59.

Bize, P., Criscuolo, F., Metcalfe, N.B., Nasir, L., & Monaghan, P. (2009) Telomere dynamics rather than age predict life expectancy in the wild. *Proceedings: Biological Sciences*, **276**: 1679-83.

Blackburn, E.H. (1991) Structure and function of telomeres. *Nature*, **350**: 569.

Blackburn, E.H., & Gall, J.G. (1978) A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *Journal of molecular biology*, **120**: 33.

Boonekamp, J.J., Mulder, G.A., Salomons, H.M., Dijkstra, C., & Verhulst, S. (2014) Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. *Proceedings: Biological Sciences*, **281**: 1-7.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., & Wittwer, C.T. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, **55**: 611-22.

Campisi, J. (2003) Cellular senescence and apoptosis: how cellular responses might influence aging phenotypes. *Experimental Gerontology*, **38**: 5-11.

Cattan, V., Mercier, N., Gardner, J.P., Regnault, V., Labat, C., Mäki-Jouppila, J., Nzietchueng, R., Benetos, A., Kimura, M., Aviv, A., & Lacolley, P. (2008) Chronic oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. *Free Radical Biology and Medicine*, **44**: 1592-98.

Cawthon, R.M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Research*, **30**.

Cawthon, R.M., Smith, K., O'Brien, E., Sivatchenko, A., & Kerber, R. (2003) Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet*, **361**: 393-95.

Cramp, S. (1988). *Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic: Vol. V. Tyrant Flycatchers to Thrushes* (Oxford University Press, Oxford, UK).

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N.B., Foote, C.G., Griffiths, K., Gault, E.A., & Monaghan, P. (2009) Real-time quantitative PCR assay for measurement of avian telomeres. *Journal of Avian Biology*, **40**: 342-47.

Foote, C.G., Gault, E.A., Nasir, L., & Monaghan, P. (2011) Telomere dynamics in relation to early growth conditions in the wild in the lesser black-backed gull: Telomeres and growth. *Journal of Zoology*, **283**: 203-09.

Fossøy, F., Johnsen, A., & Lifjeld, J.T. (2007) Multiple genetic benefits of female promiscuity in a socially monogamous passerine. *Evolution*, **62**: 145-56.

Greider, C.W., & Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell*, **43**: 405-13.

———. (1989) A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature*, **337**: 331-37.

Hall, M.E., Nasir, L., Daunt, F., Gault, E.A., Croxall, J.P., Wanless, S., & Monaghan, P. (2004) Telomere loss in relation to age and early environment in long-lived birds. *Proceedings. Biological sciences*, **271**: 1571.

Harrell Jr, F.E., Dupont, C., & others. (2020) *Hmisc: Harrell Miscellaneous.* R package version 4.4-0. URL https://CRAN.R-project.org/package=Hmisc.

Haussmann, M.F., Winkler, D.W., Huntington, C.E., Nisbet, I.C.T., & Vleck, C.M. (2004) Telomerase expression is differentially regulated in birds of differing life span. *Annals of the New York Academy of Sciences*, **1019**: 186-90.

———. (2007) Telomerase activity is maintained throughout the lifespan of long-lived birds. *Experimental Gerontology*, **42**: 610-18.

Haussmann, M.F., Winkler, D.W., O'Reilly, K.M., Huntington, C.E., & Vleck, C.M. (2003) Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings: Biological Sciences*, **270**: 1387-92.

Heidinger, B.J., Blount, B.J.D., Boner, W., Griffiths, K., Metcalfe, N.B., & Monaghan, P. (2012) Telomere length in early life predicts lifespan. *Proceedings of the National Academy of Sciences*, **109**: 1743.

Henle, E.S., Han, Z., Tang, N., Rai, P., Luo, Y., Linn, S., & Henle, E.S. (1999) Sequence-specific DNA cleavage by Fe2+-mediated fenton reactions has possible biological implications. *The Journal of biological chemistry*, **274**: 962-71.

Hol, K.E. (2018). The silver spoon effect and individual quality in relation to telomere length in blue tits (*Cyanistes caeruleus*), University of Oslo. http://www.duo.uio.no.

Hornsby, P.J. (2002) Cellular senescence and tissue aging in vivo. *The journals of gerontology. Series A, Biological sciences and medical sciences*, **57**: B251-B56.

Horváthová, T., Nakagawa, S., & Uller, T. (2012) Strategic female reproductive investment in response to male attractiveness in birds. *Proc Biol Sci*, **279**: 163-70.

Johnsen, A., & Lifjeld, J.T. (1995) Unattractive males guard their mates more closely: an experiment with bluethroats (Aves, Turdidae: *Luscinia s. svecica*). *Ethology*, **101**: 200-12.

———. (2003) Ecological constraints on extra-pair paternity in the bluethroat. *Oecologia*, **136**: 476-83.

Johnsen, A., Lifjeld, J.T., Rohde, P.A., Primmer, C., R. , & Ellegren, H. (1998) Sexual conflict over fertilizations: female bluethroats escape male paternity guards. *Behavioral Ecology and Sociobiology*, **43**: 401-08.

Johnsen, A., Pauliny, A., Lifjeld, J.T., & Blomqvist, D. (2017) Is telomere length associated with mate choice in a songbird with a high rate of extrapair paternity?

Kassambara, A. (2020) *ggpubr: 'ggplot2' Based Publication Ready Plots.* R package version 0.3.0. https://CRAN.R-project.org/package=ggpubr.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Coviello, G.M., Wright, W.E., Weinrich, S.L., & Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**: 2011-15.

Krokene, C., Anthonisen, K., Lifjeld, J.T., & Amundsen, T. (1996) Paternity and paternity assurance behaviour in the bluethroat, *Luscinia s. svecica*. *Animal Behaviour*, **52**: 405-17.

Kuznetsova, A., Brockhoff, P.B., & Christensen, R.H.B. (2017) lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, **82**: 1-26.

Lansdorp, P.M. (2005) Major cutbacks at chromosome ends. *Trends in Biochemical Sciences*, **30**: 388-95.

Macneish, A.B. (2018). Sperm telomere dynamics in a small songbird, the blue tit (*Cyanistes caeruleus*), University of Oslo. http://www.duo.uio.no.

Meyne, J., Ratliff, R.L., & Moyzis, R.K. (1989) Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proc Natl Acad Sci U S A*, **86**: 7049-53.

Monaghan, P. (2010) Telomeres and life histories: the long and the short of it. *Annals of the New York Academy of Sciences*, **12061**: 130-42.

Monaghan, P., & Haussmann, M.F. (2006) Do telomere dynamics link lifestyle and lifespan?, *Trends in Ecology & Evolution*, **21**: 47-53.

Nanda, I., Fugate, M., Steinlein, C., & Schmid, M. (2009) Distribution of (TTAGGG)n telomeric sequences in karyotypes of the Xenopus species complex. *Cytogenet Genome Res*, **122**: 396-400.

Näslund, J., Pauliny, A., Blomqvist, D., & Johnsson, J. (2015) Telomere dynamics in wild brown trout: Effects of compensatory growth and early growth investment. *Oecologia*, **177**: 1221-30.

Nettle, D., Monaghan, P., Boner, W., Gillespie, R., Bateson, M., & Bolhuis, J.J. (2013) Bottom of the heap: Having heavier hompetitors accelerates early-life telomere loss in the European starling, *Sturnus vulgaris*. *Plos One*, **8**: e83617.

Noguera, J.C., Metcalfe, N.B., & Monaghan, P. (2018) Experimental demonstration that offspring fathered by old males have shorter telomeres and reduced lifespans. *Proceedings of the Royal Society B: Biological Sciences*, **285**.

Noguera, J.C., Metcalfe, N.B., Reichert, S., & Monaghan, P. (2016) Embryonic and postnatal telomere length decrease with ovulation order within clutches. *Sci Rep*, **6**: 25915.

Oikawa, S., Tada-Oikawa, S., & Kawanishi, S. (2001) Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening †. *Biochemistry*, **40**: 4763-68.

Olovnikov, A.M. (1996) Telomeres, telomerase, and aging: Origin of the theory. *Exp Gerontol*, **31**: 443-48.

Parolini, M., Romano, A., Khoriauli, L., Nergadze, S.G., Caprioli, M., Rubolini, D., Santagostino, M., Saino, N., & Giulotto, E. (2015) Early-life telomere dynamics differ

between the sexes and predict growth in the barn swallow (*Hirundo rustica*). *Plos One*, **10**: e0142530-e30.

Pauliny, A., Larsson, K., & Blomqvist, D. (2012) Telomere dynamics in a long-lived bird, the barnacle goose. *BMC Evolutionary Biology*, **12**: 257.

Pauliny, A., Wagner, R.H., Augustin, J., Szep, T., & Blomqvist, D. (2006) Age-independent telomere length predicts fitness in two bird species. *Molecular Ecology*, **15**: 1681-87.

Petersen, S., Saretzki, G., & von Zglinicki, T. (1998) Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Experimental Cell Research*, **239**: 152-60.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**: e45-e45.

Plot, V., Criscuolo, F., Zahn, S., & Georges, J.-Y. (2012) Telomeres, age and reproduction in a long-lived reptile. *Plos One*, **7**: e40855.

Quirici, V., Guerrero, C.J., Krause, J.S., Wingfield, J.C., & Vásquez, R.A. (2016) The relationship of telomere length to baseline corticosterone levels in nestlings of an altricial passerine bird in natural populations. *Front Zool*, **13**: 1.

R Core Team. (2017). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rangbru, B. (1994). Sexual selection in the bluethroat, *Luscinia s svecica*: Relationship between male brightness and mating success - a field study, M. Sc. Thesis, University of Trondheim.

Rekdal, S.L., Anmarkrud, J.A., Lifjeld, J.T., & Johnsen, A. (2019) Extra-pair mating in a passerine bird with highly duplicated major histocompatibility complex class II: Preference for the golden mean. *Mol Ecol*, **28**: 5133-44.

Richter, T., & von Zglinicki, T. (2007) A continuous correlation between oxidative stress and telomere shortening in fibroblasts. *Experimental Gerontology*, **42**: 1039-42.

Ridout, K.K., Levandowski, M., Ridout, S.J., Gantz, L., Goonan, K., Palermo, D., Price, L.H., & Tyrka, A.R. (2018) Early life adversity and telomere length: A meta-analysis. *Molecular psychiatry*, **23**: 858.

Ringsby, T.H., Jensen, H., Pärn, H., Kvalnes, T., Boner, W., Gillespie, R., Holand, H., Hagen, I.J., Rønning, B., Sæther, B.-E., & Monaghan, P. (2015) On being the right size:

Increased body size is associated with reduced telomere length under natural conditions. *Proc Biol Sci*, **282**: 20152331.

Salomons, H.M., Mulder, G.A., Van De Zande, L., Haussmann, M.F., Linskens, M.H.K., & Verhulst, S. (2009) Telomere shortening and survival in free-living corvids. *Proceedings: Biological Sciences*, **276**: 3157-65.

Seeker, L.A., Ilska, J.J., Psifidi, A., Wilbourn, R.V., Underwood, S.L., Fairlie, J., Holland, R., Froy, H., Salvo-Chirnside, E., Bagnall, A., Whitelaw, B., Coffey, M.P., Nussey, D.H., & Banos, G. (2018) Bovine telomere dynamics and the association between telomere length and productive lifespan. *Scientific reports*, **8**: 12748.

Shay, J.W., & Wright, W.E. (1996) Telomerase activity in human cancer. *Current opinion in oncology*, **8**: 66.

Sozou, P.D., Kirkwood, T.B., & Sozou, P.D. (2001) A stochastic model of cell replicative senescence based on telomere shortening, oxidative stress, and somatic mutations in nuclear and mitochondrial DNA. *Journal of Theoretical Biology*, **213**: 573-86.

Sudyka, J., Arct, A., Drobniak, S.M., Gustafsson, L., & Cichoń, M. (2016) Longitudinal studies confirm faster telomere erosion in short-lived bird species. *Journal of Ornithology*, **157**: 373-75.

———. (2019) Birds with high lifetime reproductive success experience increased telomere loss. *Biology letters*, **15**: 20180637.

Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M.W., & Kubista, M. (2015) How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular Detection and Quantification*, **3**: 9-16.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2010) A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines. *Methods*, **50**: S1-S5.

Vedder, O., Verhulst, S., Zuidersma, E., Bouwhuis, S., & Verhulst, L. (2018) Embryonic growth rate affects telomere attrition: An experiment in a wild bird. *Journal of Experimental Biology*, **221**: urn:issn:0022-949.

Viblanc, V.A., Schull, Q., Stier, A., Durand, L., Lefol, E., Robin, J.-P., Zahn, S., Bize, P., & Criscuolo, F. (2020) Foster rather than biological parental telomere length predicts offspring survival and telomere length in king penguins. *Molecular Ecology*, **n/a**.

Voillemot, M., Hine, K., Zahn, S., Criscuolo, F., Gustafsson, L., Doligez, B., & Bize, P. (2012) Effects of brood size manipulation and common origin on phenotype and telomere length in nestling collared flycatchers. *BMC Ecology*, **12**: 17.

von Zglinicki, T. (2002) Oxidative stress shortens telomeres. *Trends in Biochemical Sciences*, **27**: 339-44.

Watson, H., Bolton, M., & Monaghan, P. (2015) Variation in early-life telomere dynamics in a long-lived bird: Links to environmental conditions and survival. *The Journal of experimental biology*, **218**: 668-74.

Watson, J.D. (1972) Origin of concatemeric T7 DNA. *Nature: New biology*, **239**: 197.

Whitlock, M.C., & Schluter, D. (2015). *The analysis of biological data* (Macmillan education: New York).

Willeit, P., Willeit, J., Mayr, A., Weger, S., Oberhollenzer, F., Brandstätter, A., Kronenberg, F., & Kiechl, S. (2010) Telomere length and risk of incident cancer and cancer mortality. *JAMA*, **304**: 69-75.

Wood, E.M., & Young, A.J. (2019) Telomere attrition predicts reduced survival in a wild social bird, but short telomeres do not. *Molecular Ecology*, **28**: 3669-80.

Zeichner, S.L., Palumbo, P., Feng, Y., Xiao, X., Gee, D., Sleasman, J., Goodenow, M., Biggar, R., & Dimitrov, D. (1999) Rapid telomere shortening in children. *Blood*, **93**: 2824-30.

Appendix

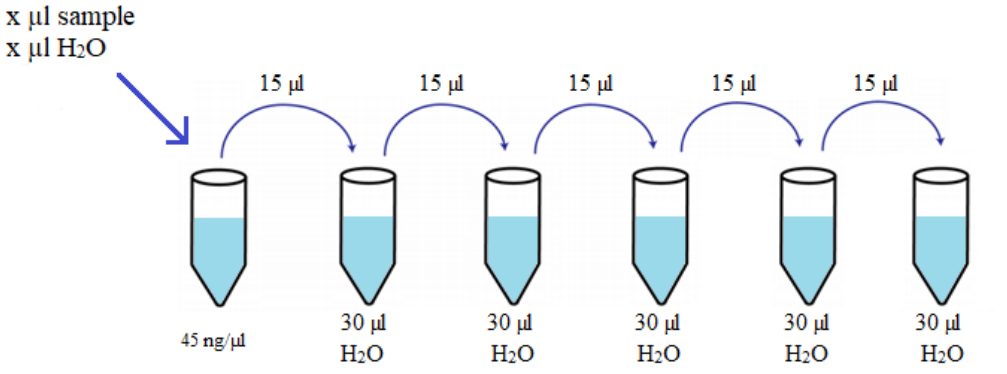
1) The qPCR programs

The table shows the programs used for qPCR for telomere- and GAPDH-amplifications of bluethroat 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

2) Standard curve serial dilutions

A 45 µl stock of 1 ng/µl and 2 ng/µl, telomeres and GAPDH respectively, was made using one DNA sample (55,4 ng/ul). The stock was then used as a basis for a 1:3 serial dilution for six replicates each with six duplicates for both concentrations. The µl volume of H_2O needed for the first tube was calculated, dependent on the concentration of each sample, using the $C_1V_1 = C_2V_2$ formula, where C_1 is the start sample DNA concentration. Thus, the volume of DNA sample and H_2O is different from standard curve to standard curve, but the total volume always makes 45 µl.



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

3) Output from CFX™ Manager

The table shows all samples used in the study included on the plate they were run on during qPCR. Yellow samples are samples in dataset 1, samples without colour are samples in dataset 2, grey samples are samples from nest 9, and the blue samples are IPC1 on the plate. Samples with a star (*) next to them had a triplicate removed to get into dataset 1.

Plate 1		Telomere		GAPDH		**Plate 2**		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.	Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		10.34	0.301	25.71	0.161	107		9.84	0.211	24.27	0.082
100026	N2U1D2	10.73	0.14	26.16	0.154	100051	N5U2D2	10.23	0.306	26.69	0.175
100027	N2U2D2	11.05	0.035	26.36	0.12	100052	N5U3D2	9.78	0.218	26.18	0.083
100028	N2U3D2	10.53	0.131	26.25	0.126	100053	N5U4D2	10.17	0.184	26.38	0.193
100029	N2U4D2	10.1	0.133	25.66	0.156	100054	N5U5D2	11.85	0.531	27.9	0.233
100030	N2U5D2	10.56	0.206	26.25	0.255	100058	N5U2D6	10.58	0.048	26.66	0.152
100031	N2U6D2	12.92	1.199	27.2	0.267	100059	N5U3D6	10.72	0.084	26.98	0.232
100032	N2U1D6	11.54	0.371	26.62	0.032	100060	N5U4D6	9.97	0.145	26.12	0.092
100033	N2U2D6	10.97	0.101	26.59	0.037	100061	N5U5D6	10.59	0.132	26.64	0.121
100034	N2U3D6	10.18	0.032	26.32	0.035	100062	N6U1D2	11.05	0.15	26.86	0.276
100035	N2U4D6	10.55	0.082	26.9	0.233	100063	N6U2D2	10.14	0.098	26.28	0.169
100036	N2U5D6	10.47	0.316	26.18	0.097	100064	N6U3D2	10.18	0.246	26.54	0.271
100037	N2U6D6	10.57	0.1	26.54	0.152	100065	N6U4D2	10.15	0.045	26.38	0.227
100038	N4U1D2	11.08	0.354	26.79	0.155	100066	N6U5D2	10	0.195	26.3	0.34
100040	N4U3D2	9.83	0.074	26.08	0.082	100067	N6U6D2	10.43	0.128	26.21	0.114
100041	N4U4D2	10.09	0.055	26.5	0.021	100068	N6U1D6	10.43	0.122	26.21	0.041
100042	N4U5D2	9.86	0.178	26.13	0.233	100069	N6U2D6	10.75	0.129	26.58	0.161
100043	N4U6D2	11.02	0.184	27.68	0.043	100070	N6U3D6	11.49	0.134	27.09	0.349
100044	N4U1D6	9.99	0.15	26.33	0.104	100071	N6U4D6	11.11	0.256	26.79	0.414
100046	N4U3D6	11.79	0.86	26.96	0.047	100072	N6U5D6	10.15	0.348	26.01	0.301
100047	N4U4D6	10.15	0.434	26.32	0.195	100073	N6U6D6	9.99	0.214	26.29	0.211
100048	N4U5D6	9.83	0.5	26.66	0.248						
100049	N4U6D6	10.28	0.342	27.45	0.18						

Plate 3		Telomere		GAPDH		Plate 4		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.	Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		10.27	0.166	25.33	0.095	107		11.16	0.702	25.85	0.164
100074	N7U1D3	10.42	0.098	25.89	0.201	100105	N10U1D2	11.34	0.112	26.46	0.175
100075	N7U2D3	10.53	0.075	26.23	0.046	100106	N10U2D2	8.77	0.321	23.91	0.072
100078	N7U1D7	10.23	0.176	26.42	0.092	100108	N10U4D2	12.46	0.263	27.5	0.025
100079	N7U2D7	10.61	0.224	26.48	0.267	100109	N10U5D2	14.66	0.47	29.88	0.174
100080	N8U1D3	10.32	0.071	26.31	0.037	100110	N10U1D6	9.86	0.196	25.65	0.078
100082	N8U3D3	10.4	0.113	26.06	0.049	100111	N10U2D6	9.84	0.2	25.96	0.102
100083	N8U4D3	9.62	0.202	25.79	0.143	100113	N10U4D6	8.44	0.201	24.12	0.053
100084	N8U5D3	10.48	0.105	26.51	0.173	100114	N10U5D6	10.77	0.219	26.56	0.169
100086	N8U7D3	9.55	0.069	25.85	0.204	100115	N11U1D2	10.97	0.151	26.83	0.136
100087	N8U1D7	10.12	0.093	27.05	0.259	100116	N11U2D2	11.03	0.142	26.57	0.109
100089	N8U3D7	10.02	0.074	26.32	0.371	100117	N11U3D2	9.09	0.185	24.52	0.103
100090	N8U4D7	10.17	0.146	26.33	0.236	100119	N11U5D2	10.42	0.371	26.1	0.314
100091	N8U5D7	9.81	0.04	26.07	0.021	100120	N11U6D2	12.03	0.272	27.37	0.114
100093	N8U7D7	10.09	0.193	26.1	0.45	100121	N11U1D6	15.55	0.28	31.48	0.58
						100122	N11U2D6	13.62	0.299	29.24	0.295
						100123	N11U3D6	13.67	0.722	28.97	0.17
						100125	N11U5D6	9.31	0.586	24.77	0.261
						100126	N11U6D6	11.27	0.503	26.77	0.128

Plate 5		Telomere		GAPDH		Plate 6		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.	Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		10.89	0.362	25.28	0.13	107		10.87	0.299	25.38	0.087
100136	N13U2D3	10.11	0.08	25.64	0.128	100147	N15U1D3	10.07	0.163	26.63	0.098
100137	N13U3D3	10.52	0.212	26.06	0.096	100153	N15U1D7	12.82	1.427	26.47	0.253
100138	N13U4D3	10.78	0.223	25.4	0.156	100177	N20U2D2	10.18	0.378	26.14	0.282
100140	N13U6D3	10.65	0.154	25.6	0.15	100180	N20U5D2	11.7	0.562	26.55	0.159
100142	N13U2D7	12.56	1.514	26.1	0.433	100183	N20U2D6	10.55	0.132	27.11	0.153
100143	N13U3D7	10.63	0.554	25.99	0.25	100186	N20U5D6	10.54	0.02	26.48	0.16
100144	N13U4D7	10.76	0.333	25.85	0.414						
100146	N13U6D7	7.55	0.204	23.32	0.326						

Plate 7		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		10.33	0.073	26.71	0.109
100160	N17U2D2	10	0.086	27.46	0.162
100161	N17U3D2	10.75	0.151	28.12	0.066
100162	N17U4D2	10.36	0.015	27.79	0.069
100163	N17U5D2	10.35	0.113	27.87	0.124
100164*	N17U6D2	9.415	0.049	27.25	0.09
100165	N17U2D6	10.29	0.125	27.78	0.132
100166	N17U3D6	9.99	0.12	27.45	0.182
100167	N17U4D6	10.78	0.073	28.4	0.098
100168	N17U5D6	10.33	0.074	27.79	0.17
100169	N17U6D6	10.56	0.162	28.28	0.192

Plate 8		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		11.22	0.182	26.11	0.052
100094	N9U1D2	11.35	0.052	27.38	0.071
100095	N9U1D3	9.54	0.144	25.41	0.172
100099	N9U1D4	10.27	0.038	26.56	0.128
100100	N9U1D5	8.62	0.145	25.15	0.106
100101	N9U1D6	11.3	0.074	27.42	0.176
100102	N9U1D7	10.975	0.035	27.06	0.027
100103	N9U1D8	11.09	0.048	27.42	0.099

Plate 9		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		10.09	0.109	26.27	0.075
100187	N21U1D6	10.51	0.336	27.29	0.098
100188	N21U2D6	10.52	0.078	27.42	0.146
100189	N21U3D6	11.03	0.111	27.58	0.137
100190	N21U4D6	10.61	0.1	27.67	0.288
100191	N21U5D6	10.68	0.26	27.95	0.136
100193	N21U1D10	10.26	0.025	27.3	0.069
100194	N21U2D10	10.56	0.111	28.18	0.051
100195	N21U3D10	10.5	0.072	28.13	0.141
100196	N21U4D10	10.28	0.054	27.74	0.15
100197	N21U5D10	10.98	0.052	27.51	0.085

Plate 10		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Mean Cq	St. dev.
107		9.9	0.048	26.26	0.093
100135	N13U1D3	9.69	0.168	26.31	0.169
100141	N13U1D7	10.03	0.145	27.26	0.111
100148	N15U2D3	9.15	0.065	26.64	0.138
100151	N15U5D3	9.9	0.055	27.02	0.097
100154	N15U2D7	9.43	0.075	26.79	0.151
100157	N15U5D7	10.06	0.152	27.32	0.11
100178	N20U3D2	9.28	0.135	27.07	0.012
100184	N20U3D6	10.21	0.018	27.67	0.152

Plate 11		Telomere		GAPDH	
Sample ID	CollNo	Mean Cq	St. dev.	Samples	St. dev.
107		11.06	0.148	26.15	0.037
100039	N4U2D2	11.03	0.076	26.39	0.097
100045	N4U2D6	11.34	0.079	26.97	0.148
100104	N9U1D9	10.41	0.179	27.04	0.11
100199	N22U1D5	11.62	0.186	28.27	0.076
100203	N22U5D5	18.61	0.126	32.23	0.71
100205	N22U1D9	12.46	0.534	27.63	0.052
100209	N22U5D9	11.89	0.58	27.42	0.093
100211	N23U2D6	10.6	0.236	27.49	0.027
100214	N23U5D6	9.61	0.479	25.43	0.092
100218	N23U2D10	11.36	0.324	28.65	0.118
100221	N23U5D10	11.24	0.164	27.1	0.077

4) Criteria for samples in the two datasets

The list shows the different criteria the samples needed to be included in the two different datasets used in the final analysis.

Criteria for samples in dataset 1	Criteria for samples in dataset 2
✓ 2 sampling days per individual nestling with a concentration over 0.25 ng/ul. - If concentration showed <0.010 ng/ul, it was set as 0.25 ng/ul after plate 8. ✓ Telomere and GAPDH worked for all triplicates on both days. ✓ Telomere and GAPDH output SD <0.22 on both 1st and 2nd sampling day. ✓ IPC on plate has SD of Cq-mean <0.22 (1st and 2nd sampling day were always on the same plate).	✓ 2 sampling days per individual nestling with a concentration over 0.25 ng/ul. - If concentration showed <0.010 ng/ul, it was set as 0.25 ng/ul after plate 8. ✓ Telomere and GAPDH got output numbers for all triplicates. × Telomere and GAPDH output SD did not have to be <0.22 on either of the sampling days. × IPC on plate did not need to have SD of Cq-mean <0.22 on either of the sampling days.

6) Variables used in the statistical analyses

The table (over next page) lists the variables that are used for the final statistical analyses in R 3.6.3., and explains their names used in the datasets. For analyses looking at rTL1, all variables from the first day were used (all variables with 1), while for analyses looking at rTL2, all variables from the second day were used. For rTL change, all variables from the first day were used, except for the mass, where the mass rate were used instead.

Name of variable	Explanation
Sample ID	The assigned number of the sample during qPCR analysis.
Plate ID	The plate ID where the sample was run during qPCR analysis.
Nestling ID	Special ID given to every individual nestling, containing nest and nestling number.
Nest ID	The nest ID assigned during the field work.
Date	Date of sampling, in June (first/second sampling day).
Time	Time of sampling converted into a numerical number (first/second sampling day).
Rain	The total downpour (millimetres) two days prior to sampling day.
Temperature	The average temperature two days prior to sampling day (first/second sampling day).
rTL first day	The relative telomere length on the first day of sampling.
rTL last day	The relative telomere length on the second day of sampling.
rTL change	The relative change in telomere length from first to second sampling (by subtracting rTL2 from rTL1).

Mass	Mass of the individual nestling on day of sampling.
Change in body mass	Difference in mass from first to second day of sampling (g).
Clutch size	Total number of eggs in the nest, unrelated if they hatch or not.
Brood size	Brood size at day of sampling.
Parental morphology	The parents' morphological traits; mass (g), tarsus length (mm) and wing length (mm)

7) Full LMER model for dataset 2

Complete LMER output, showing all the variables and their effect on relative telomere length.

Response variable	Variable	Pr(>|t|)	t	df	Estimate ± SE
rTL1	Plate	0.48	-0.72	16.7	-0.03 ± 0.042
	Date	0.34	-1	9.88	-0.058 ± 0.057
	Mass	0.92	-0.095	40.2	-0.0031 ± 0.033
	Plate	0.87	-0.17	7.57	-0.0073 ± 0.044
	Time	0.16	-1.55	8.59	-0.05 ± 0.032
	Precipitation	0.33	1.04	8.76	0.015 ± 0.015
	Temperature	0.43	0.84	7.2	0.18 ± 0.21
	Brood size	0.57	0.59	9.42	0.062 ± 0.11
rTL2	Plate	0.13	-1.6	12.9	-0.15 ± 0.091
	Date	0.68	0.43	9.29	0.059 ± 0.14
	Mass	0.94	-0.081	42	-0.006 ± 0.073
	Plate	0.22	-1.37	5.54	-0.13 ± 0.094
	Time	0.22	-1.35	7.33	-0.11 ± 0.084
	Precipitation	0.31	-1.12	5.99	-0.14 ± 0.12
	Temperature	0.97	0.042	5.09	0.017 ± 0.4
	Brood size	0.98	0.025	9.84	0.0062 ± 0.24
rTL change	Plate	0.51	-0.68	8.63	-0.076 ± 0.11
	Date	0.3	1.14	6.52	0.2 ± 0.17
	Change in body mass	0.87	-0.17	38.4	-0.028 ± 0.17
	Plate	0.42	-0.86	6.04	-0.13 ± 0.15
	Time	0.24	1.29	7.32	0.14 ± 0.11
	Precipitation	0.23	-1.31	7.51	-0.067 ± 0.05
	Temperature	0.67	-0.46	5.59	-0.33 ± 0.72
	Brood size	0.68	-0.428	8.41	-0.16 ± 0.37

Table 3. *Output from the LMER tests of all environmental factors for bluethroat nestlings, with nest ID as a random factor. Plate ID was included in all models. Significant and marginal values are marked in yellow. All n = 46.*

Response variable	Variable	Pr(>|t|)	t	df	Estimate ± SE
rTL1	Plate	0.86	0.18	8.9	0.0058 ± 0.032
	Clutch size	0.97	0.039	8.36	0.0071 ± 0.18
	Female mass	0.47	0.76	7.93	0.047 ± 0.062
	F tarsus length	0.063	2.11	9.44	0.41 ± 0.19
	F wing length	0.046	-2.28	9.96	-0.085 ± 0.037
	Plate	0.29	-1.14	8.7	-0.045 ± 0.04
	Male mass	0.87	-0.17	8.34	-0.038 ± 0.23
	M tarsus length	0.52	0.67	9.56	0.15 ± 0.23
	M wing length	0.77	0.3	8.48	0.016 ± 0.052
rTL2	Plate	0.4	-0.88	11.2	-0.058 ± 0.066
	Clutch size	0.29	-1.13	10.6	-0.42 ± 0.37
	Female mass	0.35	0.98	9.53	0.12 ± 0.12
	F tarsus length	0.026	2.52	12.9	1.03 ± 0.41
	F wing length	0.38	0.9	12.2	0.072 ± 0.08
	Plate	0.29	-1.14	8.61	-0.099 ± 0.087
	Male mass	0.86	-0.18	7.8	-0.09 ± 0.49
	M tarsus length	0.31	1.06	10.4	0.55 ± 0.51
	M wing length	0.32	-1.07	7.93	-0.12 ± 0.11
rTL change	Plate	0.23	-1.29	8.13	-0.12 ± 0.097
	Clutch size	0.59	-0.56	7.52	-0.31 ± 0.55
	Female mass	0.62	-0.52	6.84	-0.096 ± 0.18
	F tarsus length	0.8	-0.26	9.14	-0.16 ± 0.59
	F wing length	0.013	3.08	9.28	0.36 ± 0.12
	Plate	0.93	0.097	6.48	0.012 ± 0.13
	Male mass	0.93	0.091	5.99	0.065 ± 0.72
	M tarsus length	0.86	0.19	7.63	0.14 ± 0.74
	M wing length	0.33	-1.06	6.12	-0.17 ± 0.17

Table 4. *Output from the LMER tests of all parental factors on bluethroat nestlings' telomeres, with nest ID as a random factor. Plate ID was included in all models. Significant and marginal values are marked in yellow. All n = 46.*

8) Figures for Dataset 2

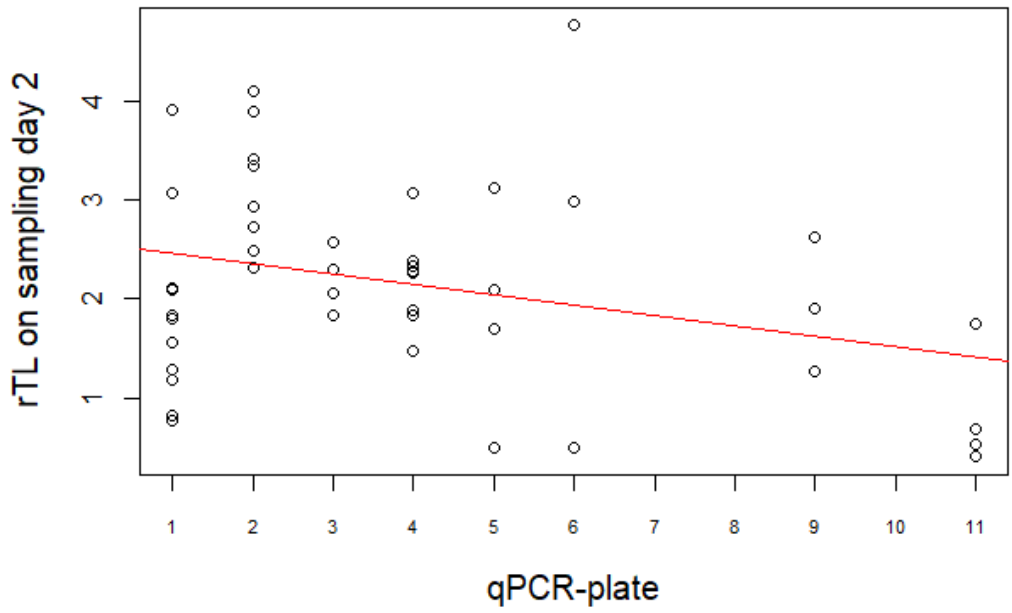


Figure 11. *Relative telomere length on sampling day 2 in relation to qPCR -plate, for bluethroat nestlings. Dataset 2, n = 46.*

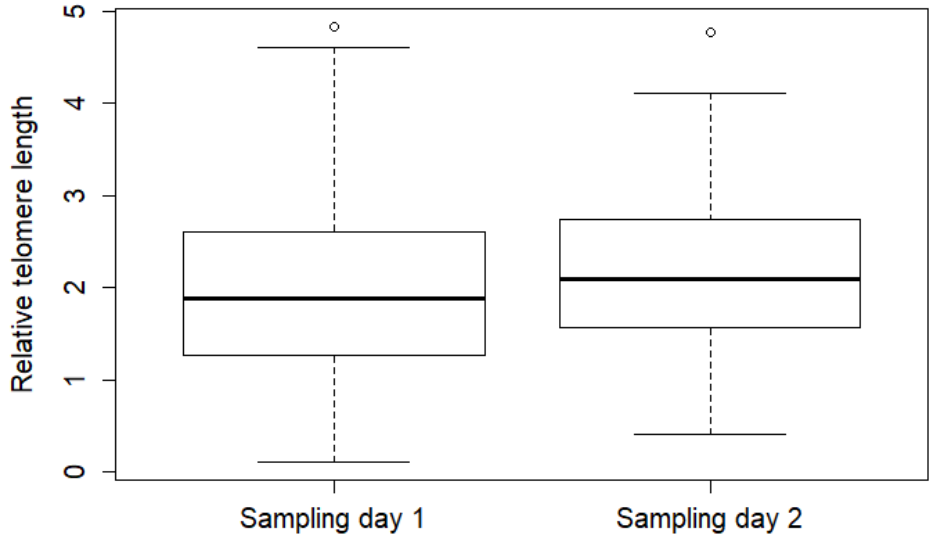


Figure 12. *Boxplot of difference in relative telomere length for bluethroat nestlings between sampling day 1 (rTL1) and sampling day 2 (rTL2). The black horizontal line shows the median (2.32 and 2.05), circles shows the outliers, and whiskers represent the lower (first) and upper (fourth) quartiles. Dataset 2, n = 46.*

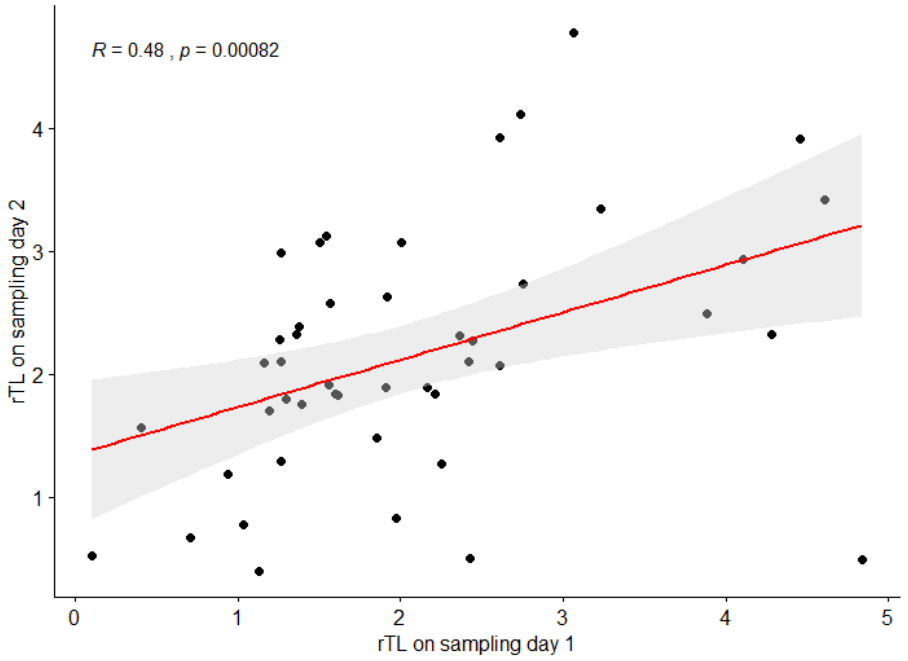


Figure 13. *Relative telomere length for bluethroat nestlings on the sampling day 2 in relation to sampling day 1. The grey area shows the 95% confidence interval. Dataset 2, n = 46.*

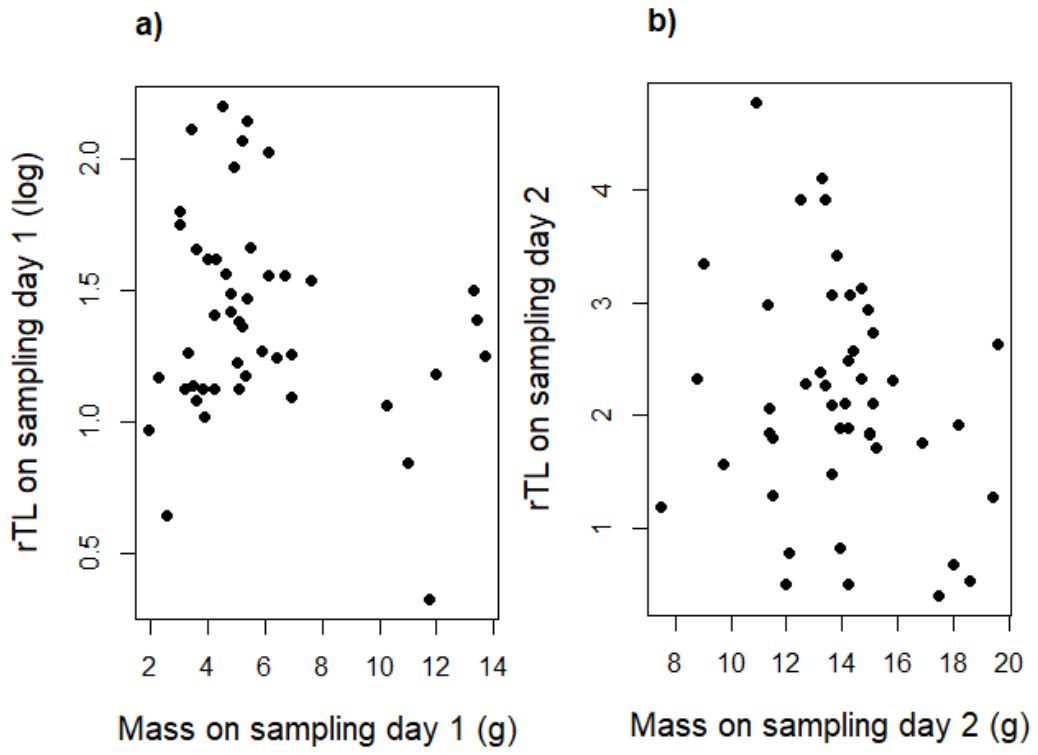


Figure 14. *Relative telomere length for bluethroat nestlings in relation to mass for a) sampling day 1, and b) sampling day 2. Dataset 2, n = 46.*

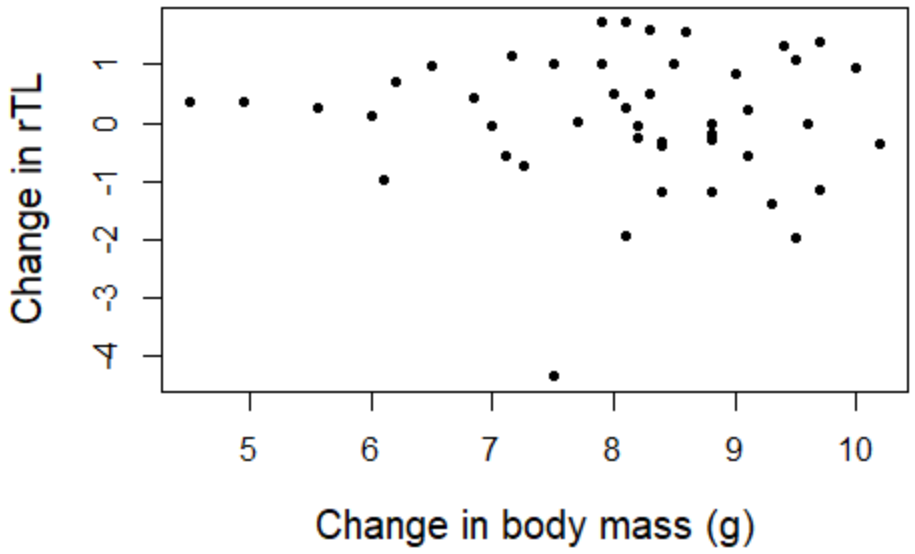


Figure 15. *Change in relative telomere length in relation to change in body mass (growth rate) for bluethroat nestlings. Dataset 2, n =46.*

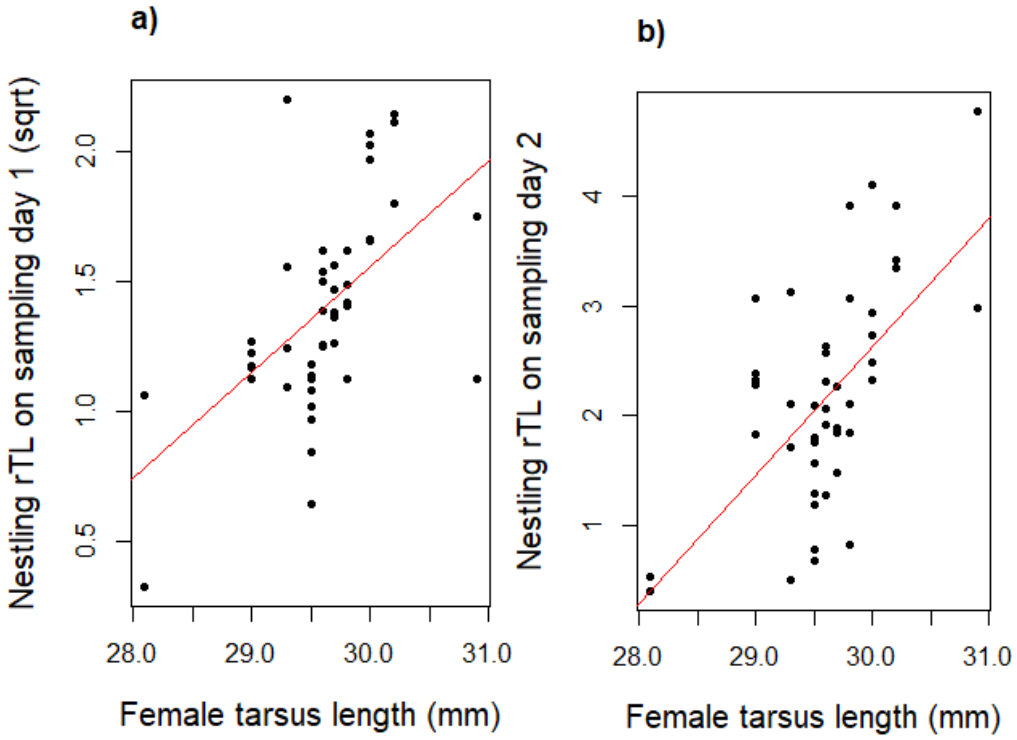


Figure 16. *Nestling relative telomere length on a) sampling day 1 and b) sampling day 2, in relation to female tarsus length, for bluethroats. Dataset 2, n = 46.*

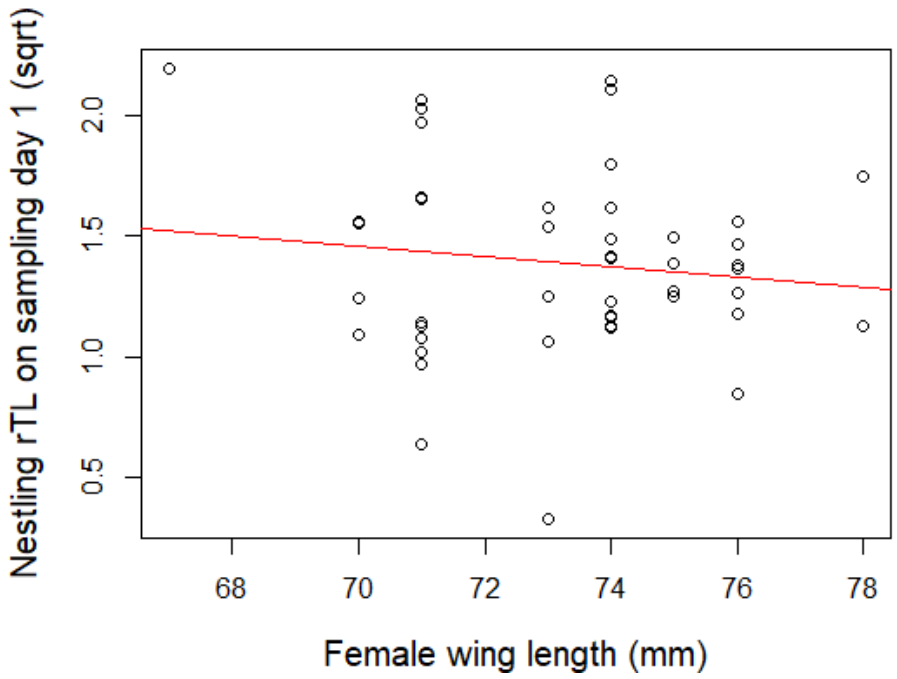


Figure 17. *Nestling relative telomere length on sampling day 1 in relation to female wing length, for bluethroats. Dataset 2, n = 46.*

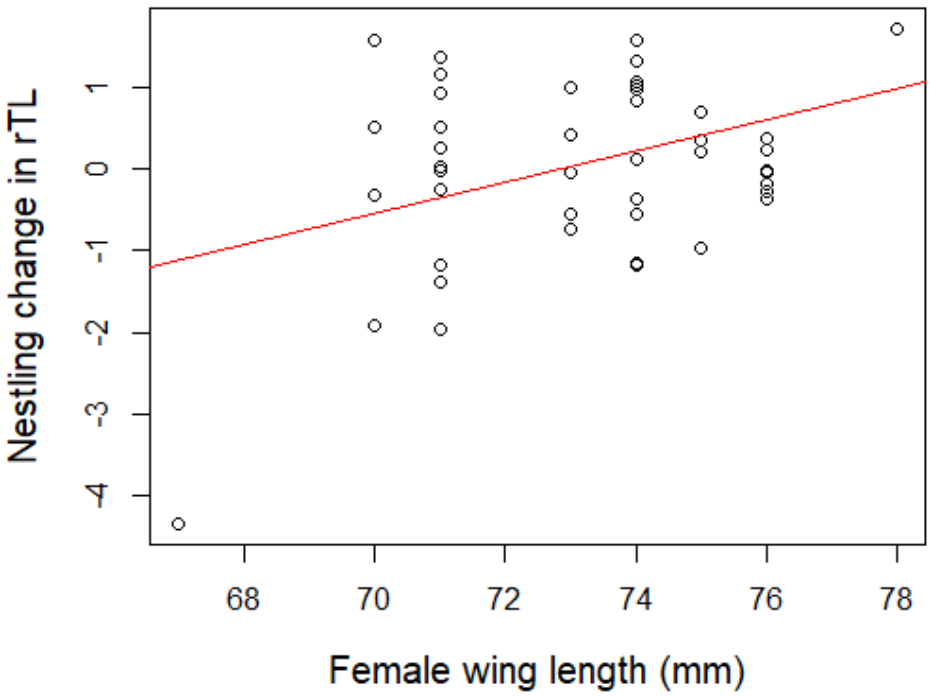


Figure 18. *Nestling relative telomere change in relation to female wing length, for bluethroats. Dataset 2, n = 46.*

9) Supplemental information on possible methodological errors, and outliers

A possible reason for the dataset being small was due to methodological and human error. The incubation time during DNA extraction might have been too long and thus degrade some of the DNA. Four plates were used for the DNA extraction, and for some of the nestlings, the sampling day 1 was extracted on a different plate than sampling day 2. Though they were all extracted with the same DNA-extraction kit, I cannot be sure that this did not have an impact on the preciseness of the results as I did not include extraction plate in my datasets. Another factor can be a variation in the pipetting technique during qPCR, as the analyses went on for longer than anticipated. This long duration of the qPCR assays might be the reason for the observed plate-effect (see Results 3.1). IPCs on four out of 11 qPCR-plates yielded a standard deviation above the accepted threshold, thus reducing the number of samples in the main dataset. Some of the calculations might also have been affected due to the standard curves yielding an E% and R2 outside the conventionally accepted range (telomeres E = 119%, R2 = 0.902; accepted upper limit E = 115%, R^2>0.980).

In dataset 1, one nestling had very long telomeres and showed the highest increase in telomere length from the first to the second sampling (Appendix 10, Chick ID N8U1). As I found no obvious error in the analysis of this sample, this seems to be a genuine change rather than a measurement error. Dataset 2 also had a nestling with a considerable change in relative telomere length (Appendix 10, Chick ID N15U1), but upon closer inspection this seems to be due to a very high Cq-mean standard deviation (~1.4), as this might have impacted the calculated values for the relative telomere length. I have chosen to include these potential outliers in the analyses.

10) Final datasets

The tables (over the next five pages) consists of the complete data set used in the analyses. The time of sampling is converted to a numeric value.

Dataset 1

ChickID	Plate (qPCR amplification)	Nest	Relative telomere length, 1st sampling	Relative telomere length, 2nd sampling	Change in relative telomere length	Body mass (g) 1st sampling	Body mass (g) 2nd sampling	Change in body mass (g)	Collection date, 1st sampling, (1 = 1 June)	Collection date, 2nd sampling, (1 = 1 June)	Time of sampling, 1st day	Time of sampling, 2dn day	Precipitation, 1st sampling	Precipitation, 2nd sampling	Temperature (°C), 1st sampling	Temperature (°C), 2nd sampling	Brood size, 1st sampling	Brood size, 2nd sampling
N4U2	11	4	1.228	1.494	0.266	3	12	9	18	22	10.5	20.5	3.54	0.48	8.43	6.05	6	6
N5U4	2	5	3.819	3.670	-0.150	5.4	11.25	5.85	17	21	15.5	12.83	0.44	0.26	7.58	6.54	5	5
N6U2	2	6	3.625	2.818	-0.807	6.2	15	8.8	18	22	9.58	20.17	3.54	0.48	8.43	6.05	6	6
N7U1	3	7	1.359	2.358	0.999	6.8	15.8	9	16	20	15.33	14	22.15	2.99	7.20	6.81	2	2
N8U1	3	8	2.021	4.144	2.123	6.4	14.9	8.5	15	19	22.23	16.25	23.54	6.25	8.32	7.55	7	7
N8U5	3	8	2.074	2.515	0.441	6.2	14.2	8	15	19	22.23	16.25	23.54	6.25	8.32	7.55	7	7
N9U1	8	9	2.364	2.535	0.170	3.9	9.9	6	19	23	22.5	21.33	6.25	0.48	7.55	6.29	1	1
N13U1	10	13	1.225	1.927	0.702	5	12.8	7.8	15	19	23.08	16.75	23.54	6.25	8.32	7.55	6	6
N15U2	10	15	2.404	2.162	-0.242	6.7	14.4	7.7	16	20	12.32	10.67	22.15	2.99	7.20	6.81	6	6
N15U5	10	15	1.779	1.969	0.190	6.2	13.5	7.3	16	20	12.32	10.67	22.15	2.99	7.20	6.81	6	6
N17U2	7	17	2.097	2.110	0.013	3.6	11.2	7.6	19	23	15.33	17.5	6.25	0.48	7.55	6.29	6	5
N17U3	7	17	1.904	2.098	0.194	2.8	8.8	6	19	23	15.33	17.5	6.25	0.48	7.55	6.29	6	6
N17U4	7	17	2.020	2.248	0.229	2.6	5.6	3	19	23	15.33	17.5	6.25	0.48	7.55	6.29	6	6
N17U5	7	17	2.147	2.064	-0.083	2.8	8.1	5.3	19	23	15.33	17.5	6.25	0.48	7.55	6.29	6	6
N17U6	7	17	2.774	2.430	-0.344	2.2	6.9	4.7	19	23	15.33	17.5	6.25	0.48	7.55	6.29	6	6
N20U3	10	20	3.008	2.282	-0.725	2.7	10.9	8.2	20	24	10	13	2.99	0.01	6.81	8.11	6	4
N21U2	9	21	1.705	2.940	1.235	10.5	14.3	3.8	20	24	12.5	18	2.99	0.01	6.81	8.11	6	6
N21U3	9	21	1.290	2.968	1.678	13.3	18.5	5.2	20	24	12.5	18	2.99	0.01	6.81	8.11	6	6

Dataset 1								
ChickID	Nest	Clutch size	Male mass (g)	Male tarsus length (mm)	Male wing length (mm)	Female mass (g)	Female tarsus length (mm)	Female wing length (mm)
N4U2	4	6	17.8	29.7	71	17.9	29.8	74
N5U4	5	7	16.4	29.9	72	23.7	30.2	74
N6U2	6	6	17.7	29.9	79	17.5	30	71
N7U1	7	6	16.8	28.4	76	17.9	29	75
N8U1	8	7	17.4	29.7	77	17.2	29.6	73
N8U5	8	7	17.4	29.7	77	17.2	29.6	73
N9U1	9	5	16.3	30.3	75	18.5	29.5	73
N13U1	13	6	17.3	30.1	78	17.6	29.3	70
N15U2	15	6	17.4	30.2	80	17	29.3	67
N15U5	15	6	17.4	30.2	80	17	29.3	67
N17U2	17	6	15.6	28.7	75	17.5	29.6	73
N17U3	17	6	15.6	28.7	75	17.5	29.6	73
N17U4	17	6	15.6	28.7	75	17.5	29.6	73
N17U5	17	6	15.6	28.7	75	17.5	29.6	73
N17U6	17	6	15.6	28.7	75	17.5	29.6	73
N20U3	20	6	17.9	30.5	77	18.5	30.9	78
N21U2	21	6	15.6	28.7	75	16.1	29.6	75
N21U3	21	6	15.6	28.7	75	16.1	29.6	75

Dataset 2																		
ChickID	Plate (qPCR amplification)	Nest	Relative telomere length, 1st sampling	Relative telomere length, 2nd sampling	Change in relative telomere length	Body mass (g) 1st sampling	Body mass (g) 2nd sampling	Change in body mass (g)	Collection date, 1st sampling, (1 = 1 June)	Collection date, 2nd sampling, (1 = 1 June)	Time of sampling, 1st day	Time of sampling, 2dn day	Precipitation, 1st sampling	Precipitation, 2nd sampling	Temperature (°C), 1st sampling	Temperature (°C), 2nd sampling	Brood size, 1st sampling	Brood size, 2nd sampling
N2U1	1	2	1.035	0.776	-0.259	3.9	12.1	8.2	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N2U2	1	2	0.937	1.188	0.251	1.95	7.50	5.55	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N2U3	1	2	1.297	1.801	0.504	3.5	11.5	8	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N2U4	1	2	1.163	2.090	0.927	3.6	13.6	10	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N2U5	1	2	1.267	1.289	0.023	3.8	11.5	7.7	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N2U6	1	2	0.407	1.566	1.158	2.55	9.7	7.15	20	24	13.42	21.5	2.99	0.01	6.81	8.11	6	6
N4U1	1	4	1.268	2.106	0.839	5.1	14.1	9	18	22	10.5	20.5	3.54	0.48	8.429	6.048	6	6
N4U3	1	4	1.976	0.825	-1.151	4.2	13.9	9.7	18	22	10.5	20.5	3.54	0.48	8.429	6.048	6	6
N4U4	1	4	2.215	1.844	-0.371	4.8	15	10.2	18	22	10.5	20.5	3.54	0.48	8.429	6.048	6	6
N4U5	1	4	2.005	3.067	1.063	4.8	14.3	9.5	18	22	10.5	20.5	3.54	0.48	8.429	6.048	6	6
N4U6	1	4	2.609	3.920	1.311	4	13.4	9.4	18	22	10.5	20.5	3.54	0.48	8.429	6.048	6	6
N5U2	2	5	4.609	3.422	-1.187	5.4	13.8	8.4	17	21	15.5	12.5	0.44	0.26	7.576	6.537	5	5
N5U3	2	5	4.459	3.907	-0.552	3.4	12.5	9.1	17	21	15.5	12.5	0.44	0.26	7.576	6.537	5	5
N5U5	2	5	3.229	3.344	0.115	3	9	6	17	21	15.5	12.5	0.44	0.26	7.576	6.537	5	5
N6U1	2	6	2.752	2.737	-0.015	5.5	15.1	9.6	18	22	9.58	20.17	3.54	0.48	8.429	6.048	6	6
N6U3	2	6	4.278	2.319	-1.959	5.2	14.7	9.5	18	22	9.58	20.17	3.54	0.48	8.429	6.048	6	6
N6U4	2	6	3.880	2.490	-1.390	4.9	14.2	9.3	18	22	9.58	20.17	3.54	0.48	8.429	6.048	6	6
N6U5	2	6	4.108	2.931	-1.177	6.1	14.9	8.8	18	22	9.58	20.17	3.54	0.48	8.429	6.048	6	6
N6U6	2	6	2.737	4.109	1.372	3.6	13.3	9.7	18	22	9.58	20.17	3.54	0.48	8.429	6.048	6	6
N7U2	3	7	1.613	1.830	0.218	5.9	15	9.1	16	20	15.33	14	22.15	2.99	7.199	6.813	2	2
N8U3	3	8	1.570	2.578	1.007	6.9	14.4	7.5	15	19	22.23	16.25	23.54	6.25	8.321	7.548	7	7
N8U4	3	8	2.595	2.478	-0.118	7.6	15.8	8.2	15	19	22.23	16.25	23.54	6.25	8.321	7.548	7	7
N8U7	3	8	2.611	2.065	-0.546	4.3	11.4	7.1	15	19	22.23	16.25	23.54	6.25	8.321	7.548	7	7
N10U1	4	10	1.378	2.387	1.008	5.3	13.2	7.9	16	20	18.77	15.75	22.15	2.99	7.199	6.813	5	5
N10U2	4	10	1.503	3.067	1.564	5	13.6	8.6	16	20	18.77	15.75	22.15	2.99	7.199	6.813	5	5
N10U4	4	10	1.258	2.284	1.026	4.2	12.7	8.5	16	20	18.77	15.75	22.15	2.99	7.199	6.813	5	5
N10U5	4	10	1.356	2.327	0.971	2.3	8.8	6.5	16	20	18.77	15.75	22.15	2.99	7.199	6.813	5	5

Dataset 2																		
ChickID	Plate (qPCR amplification)	Nest	Relative telomere length, 1st sampling	Relative telomere length, 2nd sampling	Change in relative telomere length	Body mass (g) 1st sampling	Body mass (g) 2nd sampling	Change in body mass (g)	Collection date, 1st sampling, (1 = 1 June)	Collection date, 2nd sampling, (1 = 1 June)	Time of sampling, 1st day	Time of sampling, 2dn day	Precipitation, 1st sampling	Precipitation, 2nd sampling	Temperature (°C), 1st sampling	Temperature (°C), 2nd sampling	Brood size, 1st sampling	Brood size, 2nd sampling
N11U1	4	11	2.440	2.266	-0.174	4.6	13.4	8.8	16	20	19.58	15.25	22.15	2.99	7.199	6.813	6	6
N11U2	4	11	1.912	1.890	-0.021	5.1	13.9	8.8	16	20	19.58	15.25	22.15	2.99	7.199	6.813	6	6
N11U3	4	11	1.856	1.481	-0.375	5.2	13.6	8.4	16	20	19.58	15.25	22.15	2.99	7.199	6.813	6	6
N11U5	4	11	2.162	1.887	-0.275	5.4	14.2	8.8	16	20	19.58	15.25	22.15	2.99	7.199	6.813	6	6
N11U6	4	11	1.598	1.842	0.244	3.3	11.4	8.1	16	20	19.58	15.25	22.15	2.99	7.199	6.813	6	6
N13U2	5	13	2.425	0.501	-1.924	6.1	14.2	8.1	15	19	23.08	16.75	23.54	6.25	8.321	7.548	6	6
N13U3	5	13	2.416	2.101	-0.315	6.7	15.1	8.4	15	19	23.08	16.75	23.54	6.25	8.321	7.548	6	6
N13U4	5	13	1.194	1.706	0.512	6.9	15.2	8.3	15	19	23.08	16.75	23.54	6.25	8.321	7.548	6	6
N13U6	5	13	1.539	3.123	1.584	6.4	14.7	8.3	15	19	23.08	16.75	23.54	6.25	8.321	7.548	6	6
N15U1	6	15	4.836	0.494	-4.342	4.5	12	7.5	16	20	12.32	10.67	22.15	2.99	7.199	6.813	6	6
N20U2	6	20	3.059	4.772	1.713	3	10.9	7.9	20	24	10	13	2.99	0.01	6.813	8.111	6	4
N20U5	6	20	1.265	2.983	1.719	3.2	11.3	8.1	20	24	10	13	2.99	0.01	6.813	8.111	6	4
N21U1	9	21	1.558	1.910	0.353	13.7	18.2	4.5	20	24	12.5	18	2.99	0.01	6.813	8.111	6	6
N21U4	9	21	1.921	2.625	0.704	13.4	19.6	6.2	20	24	12.5	18	2.99	0.01	6.813	8.111	6	6
N21U5	9	21	2.248	1.272	-0.976	13.3	19.4	6.1	20	24	12.5	18	2.99	0.01	6.813	8.111	6	6
N22U1	11	22	1.126	0.401	-0.726	10.25	17.5	7.25	20	24	16.58	22.5	2.99	0.01	6.813	8.111	5	5
N22U5	11	22	0.105	0.524	0.419	11.75	18.6	6.85	20	24	16.58	22.5	2.99	0.01	6.813	8.111	5	5
N23U2	11	23	1.390	1.752	0.362	11.95	16.9	4.95	20	24	21.62	22	2.99	0.01	6.813	8.111	7	7
N23U5	11	23	0.711	0.675	-0.036	11	18	7	20	24	21.62	22	2.99	0.01	6.813	8.111	7	7

Dataset 2																		
ChickID	Nest	Clutch size	Male mass (g)	Male tarsus length (mm)	Male wing length (mm)	Female mass (g)	Female tarsus length (mm)	Female wing length (mm)	ChickID	Nest	Clutch size	Male mass (g)	Male tarsus length (mm)	Male wing length (mm)	Female mass (g)	Female tarsus length (mm)	Female wing length (mm)
N2U1	2	6	16.6	29.7	77	18.2	29.5	71	N11U1	11	6	18.4	30.7	77	18.2	29.7	76
N2U2	2	6	16.6	29.7	77	18.2	29.5	71	N11U2	11	6	18.4	30.7	77	18.2	29.7	76
N2U3	2	6	16.6	29.7	77	18.2	29.5	71	N11U3	11	6	18.4	30.7	77	18.2	29.7	76
N2U4	2	6	16.6	29.7	77	18.2	29.5	71	N11U5	11	6	18.4	30.7	77	18.2	29.7	76
N2U5	2	6	16.6	29.7	77	18.2	29.5	71	N11U6	11	6	18.4	30.7	77	18.2	29.7	76
N2U6	2	6	16.6	29.7	77	18.2	29.5	71	N13U2	13	6	17.3	30.1	78	17.6	29.3	70
N4U1	4	6	17.8	29.7	71	17.9	29.8	74	N13U3	13	6	17.3	30.1	78	17.6	29.3	70
N4U3	4	6	17.8	29.7	71	17.9	29.8	74	N13U4	13	6	17.3	30.1	78	17.6	29.3	70
N4U4	4	6	17.8	29.7	71	17.9	29.8	74	N13U6	13	6	17.3	30.1	78	17.6	29.3	70
N4U5	4	6	17.8	29.7	71	17.9	29.8	74	N15U1	15	6	17.4	30.2	80	17	29.3	67
N4U6	4	6	17.8	29.7	71	17.9	29.8	74	N20U2	20	6	17.9	30.5	77	18.5	30.9	78
N5U2	5	7	16.4	29.9	72	23.7	30.2	74	N20U5	20	6	17.9	30.5	77	18.5	30.9	78
N5U3	5	7	16.4	29.9	72	23.7	30.2	74	N21U1	21	6	15.6	28.7	75	16.1	29.6	75
N5U5	5	7	16.4	29.9	72	23.7	30.2	74	N21U4	21	6	15.6	28.7	75	16.1	29.6	75
N6U1	6	6	17.7	29.9	79	17.5	30	71	N21U5	21	6	15.6	28.7	75	16.1	29.6	75
N6U3	6	6	17.7	29.9	79	17.5	30	71	N22U1	22	5	16.3	28.6	76	16.4	28.1	73
N6U4	6	6	17.7	29.9	79	17.5	30	71	N22U5	22	5	16.3	28.6	76	16.4	28.1	73
N6U5	6	6	17.7	29.9	79	17.5	30	71	N23U2	23	7	16.6	30	76	18.4	29.5	76
									N23U5	23	7	16.6	30	76	18.4	29.5	76