A longitudinal analysis of telomeres in an insular house sparrow population

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Abstract

Telomeres show variation in length, both between and within species, and have been proposed as a biomarker of biological ageing, providing information on the current physiological state of individuals, and reflecting (accumulated) somatic damage over time. The underlying causes of variation in telomere length remain unclear. In this thesis, I studied telomere dynamics in a wild passerine population. First, I assessed the methodological consequences of storage of blood in ethanol for the subsequent measurement of telomere length using qPCR. I found that telomere length estimates shorten in a quadratic relationship when blood samples are stored for relatively long periods. I then show that telomeres shorten with age within individuals, and that individuals with shorter telomeres disappear more quickly from the population. Moreover, individuals showed low repeatability of telomere length over time, with some individuals showing significant elongation of telomeres.

Using pedigree information, I then estimated the heritability of telomere length, and concluded that this is low in house sparrows, and the variation in telomere length of offspring was explained more by the year of birth. In addition, the age of the biological father had a negative effect on offspring telomere length. I also found that the age of the rearing male had a positive effect on offspring telomere length, but only at the population level. This, again, indicates the selective disappearance of individual adults and/or offspring with shorter telomeres.

In conclusion, my results show that telomeres may not reflect accumulated damage but may instead be more plastic than previously thought. Telomeres may instead reflect more immediate circumstances of the individual. Regardless, individuals overall still showed significant telomere attrition with age, and individuals with relatively short telomeres disappeared from the population, supporting the use of telomeres as a biomarker of ageing in wild passerines.

Acknowledgements

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I would also like to thank Mirre Simons for providing the statistical knowledge that was needed to complete my PhD. I may not always have been able to immediately grasp the statistics you explained in so much detail, but your explanations were immensely helpful, so thank you for having patience with me.

And then I would like to thank Julia Schroeder for providing valuable insights in heritability, and for reading many of my chapters. Our fieldwork wintertrips to Lundy, organised in detail by you to accommodate a great number of master students, were a real treat. You provided valuable feedback that I used to improve my work.

Many thanks to Nathalie Dos Remedios and Marie-Elena Mannarelli for measuring all the telomeres lengths in thousands of samples and providing me with knowledge on how to measure telomere length on the qPCR.

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And of course, a great thanks to my family, who were supportive right from the start, even when they realized I would be living abroad on an island, and then stay 3 to 4 months each year on an even smaller and completely remote island.

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A group of young and old house sparrow males, with coloured bands, on a rooftop on Lundy Island.

1. General introduction

1.1 Senescence

Senescence, or the deterioration of a phenotype and a reduction of residual reproduction at older ages, occurs in most organisms (Møller 2006; Nussey *et al*. 2013). Previously, it was assumed that wild animals do not experience senescence due to high extrinsic mortality rates (Barrett *et al*. 2013). However, evidence that animals do experience senescence has accumulated over the years (Barrett *et al.* 2013; Nussey *et al.* 2013). Evidence of senescence is shown by an age-specific pattern of phenotypic improvement in early life, followed by a plateau and decline later in life. Why humans and animals experience senescence has been a central question for a long time (Pauliny & Blomqvist 2012). After all, senescence seems maladaptive because individuals with an increased lifespan will also have more reproductive opportunities (Haussmann *et al*. 2003), and as such would have a selective advantage over those with shorter lives. Yet a decline in both survival and reproduction occurs in many species (Williams 1957). This seems like an evolutionary paradox.

There are three main theories why senescence occurs, all based around the concept that selection favours early-life reproduction and is thus strongest in early life (Hammers *et al.* 2015). The first one, the antagonistic pleiotropy hypothesis, states that genes that have a positive effect in early life can have a deleterious effect later in life but can still be favoured due to the net benefit of the positive early-life effects (Hamilton 1966, Kirkwood & Austad 2000). The second theory is the mutation accumulation hypothesis. This hypothesis postulates that deleterious mutations that act late in life accumulate over evolutionary time, because selection is weaker at older ages due to a combination of mortality and lower remaining lifespan (Medawar 1952). This accumulation of genes that only have a negative effect late in life may be the cause of senescence. The third theory is the disposable soma theory. It was first proposed by Weismann (1892), who suggested that senescence is part of the trade-off framework. Trade-offs occur because animals have restricted resources to spend on different life-history components, such as reproduction and (somatic) maintenance (Stearns 1976). Investing energy in one component means that the same energy cannot be invested into another. Trade-offs are visualised with fitness as the paid currency, where one trait negatively influences the fitness outcome from another trait (Stearns 1989). This theory predicts that, because mortality increases with age, reproduction early in life is favoured, but comes at the cost of somatic maintenance. Senescence evolves as a consequence of this selection favouring early-life reproduction.

Because of the trade-offs between maintenance and reproduction, the senescent phenotype is also influenced by individual quality. High-quality individuals may have access to more resources than low-quality individuals, and are thus less impacted by the 'choice' between maintenance, reproduction and survival (van Noordwijk & de Jong 1986). Qualitative differences between individuals lead to differences in fundamental trade-off because individuals show variation in how much they 'suffer' in terms of costs they endure and benefits they accrue at a given level of reproductive investment (Stearns 1992). Such qualitative effects may make the detection of life-history trade-offs difficult, as they can mask traded-off costs (Nussey *et al*. 2013).

Variance in individual quality, among other factors, may thus affect how individuals are impacted by trade-offs and, consequently, lead to variation in life-history traits. The variation between individuals and resulting consequences on the rate of senescence led to the attempt to identify the actual molecular or physiological causes of senescence. From laboratory experiments, one of the proposed mechanisms that underlie age-related declines in physiological function were telomeres.

1.2 Telomeres and senescence

Telomeres are composed of DNA with hundreds of copies of the repeated sequence 5'TTAGGG-3 (Meyne *et al.* 1989). Telomeres are located at the ends of chromosomes (Blackburn 1991). Telomeres are double stranded, but the end is a single-stranded G-rich overhang. This G-rich extension loops back on itself and back into the double-stranded telomeric DNA part. This t-loop needs to be maintained to prevent DNA damage, which is done by proteins such as the shelterin and CST complexes (Monaghan *et al.* 2018).

Telomeres have several functions within a cell: 1. Telomeres safeguard chromosome integrity by protecting chromosomes from nucleotide loss, as the ends of chromosomes are not fully replicated during cell division (Blackburn 1991; Monaghan *et al*. 2018). This 'end replication problem' would be detrimental for DNA, and thus telomeres shorten instead. 2. Protecting DNA from cellular stresses, such as oxidative stress, which also causes telomeres to shorten (Bize *et al.* 2009, Blackburn *et al.* 2015; Reichert *et al.* 2017). 3. When telomeres become critically short, they cause a cellular signal, after which cells can suffer from DNA damage, leading to apoptosis (Blackburn 1991; von Zglinicki 2002). So telomeres serve a central role in genomic integrity, but as a consequence shorten, which may have its own consequences. Critically short telomeres may increase local inflammation (Campisi *et al*. 2005), cause genomic instability (Kong *et al*. 2013), and reduce vascular function and health

(Sharpless & DePinho 2004). Moreover, short telomeres have been associated with lower survival in birds (Wilbourn *et al*. 2018).

Telomeres will be the focus in this thesis to assess their links with life-history traits. I use a closed and wild population of house sparrows, *Passer domesticus*, on Lundy island (see next section). Such an insular population gives valuable insights into telomere dynamics both within a population as within individuals (see box 3). This longitudinal research is needed because even though longitudinal studies of telomere dynamics are emerging, most studies are still focused on cross-sectional effects (Monaghan *et al*. 2018, also see Box 1.3).

1.3 House sparrows on Lundy

We collected blood samples from adult house sparrows located on the small island of Lundy, in the south-west of England (Figure 1.1), in the years 2000-2016. Ninety-nine per cent of the house sparrows in our population are banded (Schroeder *et al*. 2015). House sparrows are not well suited for long-distance flights (Bengtson *et al*. 2004) and due to the distance to the mainland (18 km), dispersal is nearly non-existent (Schroeder *et al*. 2015). In our population, data from 13 microsatellite markers was used to assign the parentage of the small number of unbanded birds that were not observed as chicks in the nest (Schroeder *et al*. 2015). From this analysis, it was concluded that immigration accounted for *ca.* 0.5% of all recruits (Schroeder *et al.* 2015). Moreover, it was concluded that the probability of re-sighting an individual on Lundy was between 0.91 and 0.96 (Schroeder *et al*. 2015; Simons *et al.* 2015a). This means that we had available detailed lifespan and survival information, as well as the exact known ages of individuals that would normally be difficult to obtain in the wild.

Figure 1.1. Lundy island (red circle), located in the Bristol Channel. Upper left: a banded Lundy House sparrow female.

1.4 Measuring telomeres

Telomeres can be measured using a variety of methods. One of the older methods is the Telomere (terminal) Restriction Fragment method, or TRF (Criscuolo *et al*. 2009). In this method non-telomeric DNA is digested, after which a phosphorescent or radioactive probe is used to measure the mean lengths of TRFs using a Southern blot or in-gel hybridization (Nakagawa *et al*. 2004). This method is relatively easy and provides inter- and intra-gel coefficients of variation among individual birds of less than 2% (Haussmann *et al.* 2003). It also provides measurements of absolute telomere lengths (Cawthon *et al*. 2002).

There are a few drawbacks of the TRF method. For one, autoradiographic smears may not be straightforward to interpret (Criscuolo *et al*. 2009). Moreover, this method requires relatively large amounts of DNA (5-10 μg), obtained from carefully preserved tissue samples, and is time consuming (Haussmann & Mauck 2008).

These drawbacks can be avoided with a newer method: quantitative PCR, or qPCR (see Box 1.1). This method is now the most popular for measuring telomere length (Martin-Ruiz 2015). In qPCR,

primers are used to amplify random telomere repeats, with the amount of the product being proportional to the total telomere length (Cawthon 2009). The qPCR method also has a few drawbacks: 1. It measures a *T/S* ratio, where telomere length (*T*) is expressed in proportion to a control gene (*S*) (Cawthon 2002). 2. Interstitial repeats are also incorporated into the amplification, and can thus cause a bias in animals that have large numbers of interstitial repeats, or large variation of interstitial repeats between individuals (Nanda *et al.* 2002; Barrett *et al*. 2012).

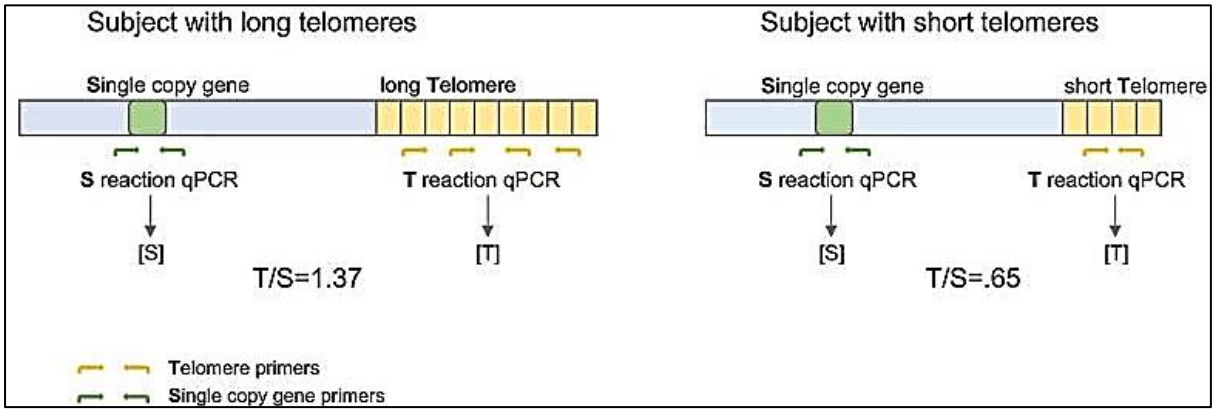
We chose to analyse our house sparrow blood samples with qPCR for a multitude of reasons: 1. Sample measurement speed. qPCR analysis is seven times faster than TRF. With over 2,500 samples in total to analyse, with hundreds of samples added each year, sampling efficiency was important. 2. Even though the avian genome has many interstitial repeats (Nanda *et al.* 2002) and the qPCR method detects these repeats alongside the telomeres, these repeats do not change with age (Delany *et al.* 2003). This means that the inclusion of these telomeric repeats should not impact on the conclusions concerning telomere dynamics (but see Foote *et a*l. 2013). 3. Results from the TRF and qPCR methods have been shown to be highly correlated, and thus qPCR is a reliable method (Criscuolo *et al*. 2009).

Box 1.1: Measuring telomeres with Quantitative Polymerase Chain Reaction (qPCR)

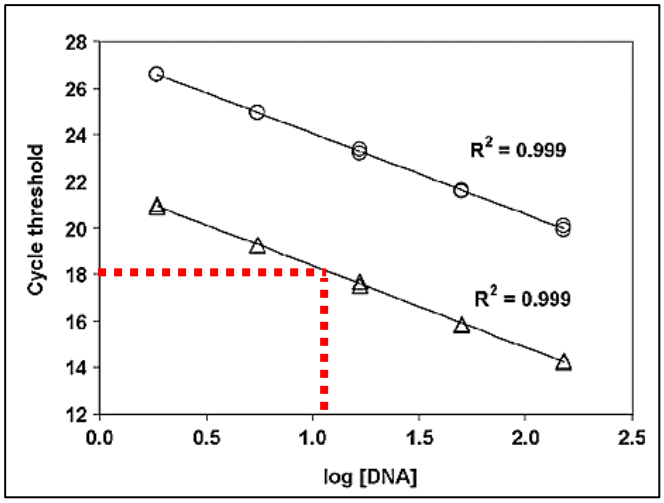
qPCR monitors the amplification of DNA in real time, by measuring the total fluorescence after each cycle of amplification using fluorescently labelled primers (Holland *et al.* 1991; Higuchi *et al.* 1992). These primers attach themselves to the DNA sequence of interest, which allows the amount of DNA to be measured via the measurement of the fluoresce. Multiple DNA sequences labelled with different dyes can be measured at the same time (Cawthon 2009). We used the telomeric DNA (*T*) and a reference gene, *GAPDH* (*S*), measured in the same well on a qPCR plate and then calculated the *T/S* ratio to normalise the telomere estimate among samples. See Figure A below, which shows an example of a subject with long and short telomeres (taken from Lin *et al.* 2019).

After measuring the fluorescence, the quantity of DNA at the start of amplification is still unknown. To calculate the amount of DNA that was available before amplification, standards dilutions are used. These dilutions of known concentrations are also amplified and measured on the qPCR. The logarithms (base 10) of 5 different concentrations are then plotted. The amount of DNA present in a sample can then be calculated in reference to the known concentrations of the standards (Cawthon 2009). See Figure B, which shows an example (red dotted lines) how the amount of (*T*) is calculated when the fluorescence is detected after 18 cycles, leading to a starting DNA concentration of 1.1 (taken from Cawthon 2009).

A (from Lin *et al.* 2019)



B (from Cawthon 2009)



1.5 Telomeres and sample storage

With an increase in studies that focus on measuring telomeres, methods regarding collecting samples, extracting DNA and analysing telomeres have undergone scrutiny (Nussey *et al.* 2014). Because telomeres are located at the end of chromosomes, degradation of DNA over time is a potential issue that will affect telomeres first (Nussey *et al*. 2014).

There are different ways to store blood samples, such as storage in ethanol or on FTA cards (Hatman Flinders Technology Associates). There are only a few studies that have investigated storage methods, but the overall conclusions were that telomere length measurement by qPCR may indeed be influenced by the storage method (Zanet *et al*. 2013, Reichert *et al*. 2017a; but see Tolios *et al.* 2015). Also, within the same storage method, differences can potentially accumulate between samples that are stored for a short or long time. This is a potential issue, especially in long-term and longitudinal datasets. Longitudinal datasets utilise a time-series, measuring individuals multiple times. Any conclusions regarding telomere loss during such a time series may not be meaningful if the earlier collected samples have degraded over time.

I was interested in studying long-term telomere dynamics in our house sparrow population. But upon initial investigation of our data, I noticed a decline in telomere length at older years that could not be explained by the age of individuals. Surprisingly, only a few studies have investigated effects of storage time on telomere length. Most studies focus on the method of storage, or only investigated short term storage effects on telomere length. Because I needed to understand the relationship between storage time and telomere length, we set up an experiment to assess if the decline was due to blood sample or DNA storage time.

1.6 Telomere attrition and elongation

The first study that studied telomere length observed that telomeres shortened in cultures of human fibroblasts (Harley *et al.* 1990). After the discovery that telomeres shorten with each cell division until a critical end, telomeres were proposed as a mitotic clock (Olovnikov 1996). Such a mitotic clock suggested that telomeres shortened at a constant rate (Haussmann & Vleck 2002; Ju & Rudolph 2008). The large body of work that has subsequently studied the change in telomere length with age has indeed concluded that telomeres shorten in many species (Dantzer & Fletcher 2015). However, if telomeres shorten at a constant rate, variation in telomere length between individuals of the same age and species should be very low. Instead, there is high variation in telomere length among individuals of the same species and age (Monaghan & Haussmann 2006; Hares *et al.* 2018).

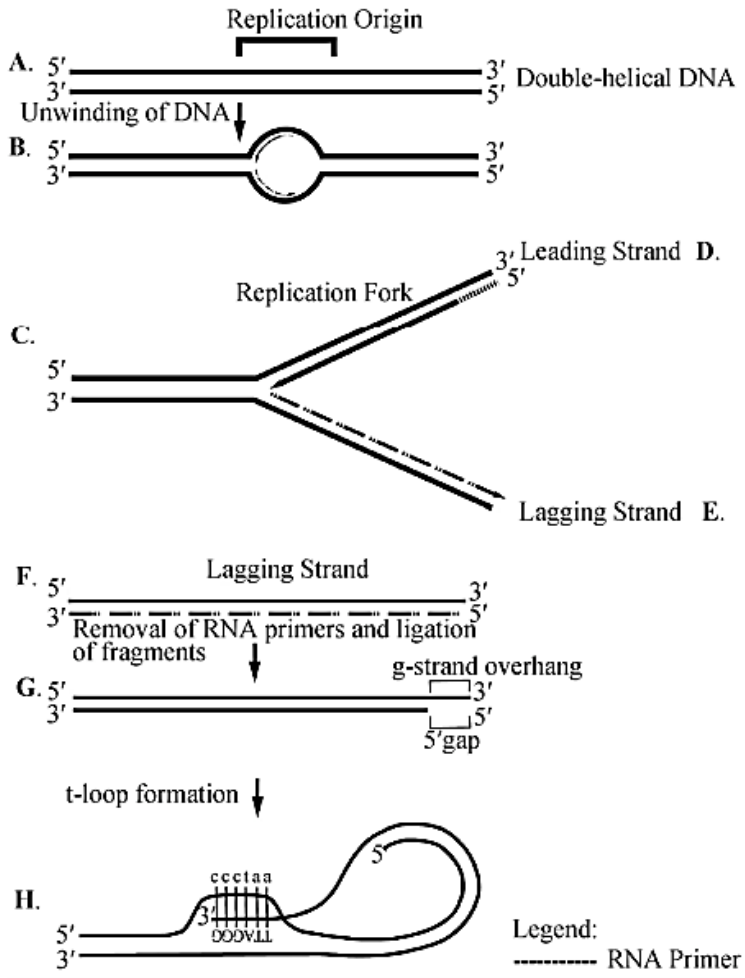
The observed variation in telomere length can occur due to three, non-mutually exclusive causes: 1. Environmental effects which affect the amount of (oxidative) stress that individuals experience, which in turn affect telomere attrition (Von Zglinicki 2002). 2. Telomere restoration processes, such as telomerase. 3. Initial telomere length of the zygote, determined by, for example, genetic or parental factors, meaning that individuals start life with different telomere lengths (Olsson *et al.* 2010), discussed in Section 1.8 and Chapters 4 and 5.

Telomeres can shorten due to different processes. The loss of telomeric base pairs with each cell division is due to an incomplete replication process, called the end-replication problem (Box 1.2). Telomeres can shorten between 30 to 200 base-pairs per cell division, however, in humans only 10 base-pairs are attributable to the end replication problem (von Zglinicki *et al*. 2001). This means that the remaining telomeric losses must be due to other processes. The most important factor causing telomere shortening is oxidative stress (Houben *et al.* 2008; Monaghan 2014; Zhang *et al*. 2015; Reichert *et al*. 2017).

Oxidative stress arises when reactive oxygen species (ROS) are generated. ROS are, among other things, a by-product of aerobic metabolism and ATP production in mitochondria and are highly reactive (Balaban *et al*. 2005). ROS are both non-radical oxidants and free radicals. Both non-radical oxidants and free radicals are unstable and can cause chain reactions within cells that eventually can lead to damage (Monaghan *et al.* 2009). Telomeres, having a high guanine content and are therefore specifically susceptible to oxidative damage (Henle *et al*. 1999). The effects of ROS have been intensively studied *in vitro,* where oxidative stress affects telomere length in a dose-dependent manner (von Zglinicki 2002).

Organisms have evolved mechanisms to prevent or repair oxidative damage (Haussmann & Marchetto 2010). Oxidative damage is therefore a balance between free radicals and defence mechanisms (Haussmann & Marchetto 2010). When the production of ROS then exceeds the defence mechanisms, oxidative damage still occurs (Monaghan *et al*. 2009). Oxidative stress occurs in animals that are stressed, producing stress hormones, such as cortisol and corticosterone. Such hormones elicit a reallocation of resources to maximize survival (Haussmann & Marchetto 2010). However, cortisol and corticosterone hormones are also linked to higher oxidative stresses and have been shown to cause telomere attrition in vitro (Steptoe *et al*. 2017).

Box 1.2: The end-replication problem



A. The double stranded helix is unwound by enzymes.
B. As the strands separate, two replication forks form in opposing directions.
C. One half of the replication fork is shown.
D. The leading strand demonstrates continuous replication because DNA polymerase can only synthesize new DNA in the direction 5'->3'.
E. The lagging strand carries out discontinuous replication producing small fragments and requiring multiple RNA primers.
F. After DNA polymerase synthesizes the new fragments, exonuclease must remove the RNA primers and the fragments are ligated together.
G. DNA polymerase is unable to fill in the 5' gap.
H. The g-strand overhang is tucked into a protective t-loop structure.

Taken from Haussmann & Marchetto 2010

However, *In vivo*, conclusions regarding associations between oxidative stress and telomere length are ambiguous in correlative studies. From 18 studies that studied possible correlations between stress hormones and telomere lengths in humans and birds, 10 reported a significant correlation between stress hormones and telomeric loss (Reichert *et al*. 2017). Experimental studies were less ambiguous. When experiments where stress hormones were experimentally controlled were conducted, seven out of eight studies provided support for oxidative stress causing telomere shortening (Reichert *et al*. 2017). However, this link appears to be dependent on factors such as tissue type or sex. Such a dependence does explain how variation in telomere length exists if, for example, sexes respond differently to stresses and as a consequence, show a difference in telomere length.

Regardless of not knowing the exact mechanisms that mediate telomere loss, it is evident that animals experience environmental stresses in the wild, that in turn cause stress hormone and/or oxidative stress to shorten telomeres. Because older individuals would have endured more stresses in their lives, leading to damage in telomeres, a link between biological age and telomere length was proposed. Telomeres were seen as biomarkers of age, as they could reflect the cumulative damage an individual had endured during its lifetime (Zglinicki & Martin-Ruiz 2005). Many studies in various species found that telomeres indeed shorten with age (overview in Dantzer & Fletcher 2015). But more recent studies show that telomeres may not only shorten, but can also elongate (Bateson & Nettle 2017; Spurgin *et al*. 2018; Fairlie *et al.* 2016). Telomeres can be repaired and even lengthen after damage (Spurgin *et al.* 2018). Telomere increases can occur due to changes in cellular composition or due to telomeres elongating via telomerase (Blackburn *et al*. 1989; Spurgin *et al*. 2017).

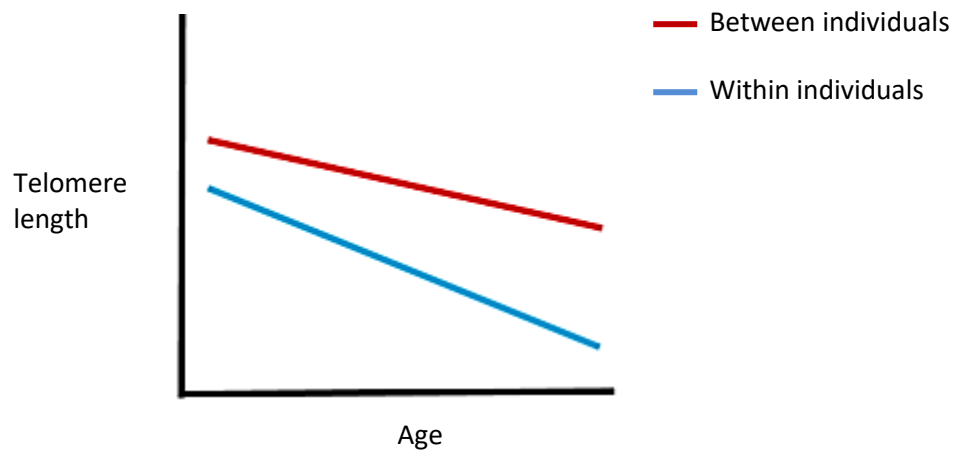
Telomerase is found in all organisms that have nucleoid cells with only very few exceptions (Greider & Blackburn 1996). It solves the end replication problem by adding DNA to chromosomes before replication starts, resulting in daughter strands that are as long as the parent strands (Greiger & Blackburn 1996). Telomerase may pose as the ultimate solution to maintain cell integrity indefinitely. However, cells that would endlessly replicate are at a risk to become malignant (Monaghan & Haussmann 2006). This chance of malignancy is shown by the result that telomerase has been associated with cancer, caused by unlimited cell proliferation (Greiger & Blackburn 1996; Monaghan & Haussmann 2006). This increased chance of malignancy is likely the reason why telomerase activity is mostly found (although not exclusively) in germlines and embryos (Monaghan & Haussmann 2006).

Regardless, elongation of telomeres has been found in some studies (Bateson & Nettle 2017; Spurgin *et al*. 2018; Fairlie *et al*. 2016). Telomere elongation, especially when influenced by environmental conditions, would suggest telomere length may not reflect accumulated costs accurately (Bateson 2016). Instead, telomeres may be biomarkers of the body's ability to absorb oxidative damage (Boonekamp *et al.* 2013).

Such new insights regarding the telomere dynamics and individual age effects only came to light when individuals were measured more than once. Previously, many of the correlational links between age and telomere length were from cross-sectional datasets. The cross-sectional design can only infer the telomere shortening based on the telomere lengths of individuals of different ages (Chen *et al*. 2011). In more recent years, longitudinal studies did start to emerge. These studies managed to look more precisely at within-individual effects and some concluded that the attrition of telomeres both between and within individuals is variable (Spurgin *et al.* 2018, Atema *et al*. 2019). In a cross-sectional population, age effects that are found at the population level can be confounded by environmental effects on the population, such as those that influence entire cohorts, which affect age specific effects (Hall *et al*. 2004). Age classes can also be affected by the selective disappearance of certain individuals with age (Nussey *et al*. 2011, Lindström 1999). The latter is called selective disappearance.

Selective disappearance occurs when individuals that show a trait disappear out of the population. Selective disappearance can be detected in longitudinal studies when a within-individual decline of telomere length occurs, while between-individuals, a decline (or a less steep decline) is not detected (Barrett *et al*. 2013; Box 1.3). Selective disappearance can also be detected when between-individuals there is a positive age effect, while within-individuals there is a negative or no effect.

Box 1.3: Cross-sectional versus longitudinal and selective disappearance



Cross-sectional data measures an individual once and can thus only detect a between-individual effect, indicated with the red line in the Figure above. Longitudinal data measures an individual multiple times, and can thus assess changes within individuals, indicated with the blue line in the Figure above.

To detect selective disappearance, between-individual effects need to be compared to within-individual effects. When, as seen in the example in the Figure, the between individual effect show a less steep decline of telomere length with age than the within-individual effect, selective disappearance has to act within the population. After all, a negative effect that occurs within-individuals cannot be less strong between-individuals unless individuals that show a relatively strong effect of age on telomere length disappear out of the population.

1.7 Telomeres and survival

A meta-analysis in humans revealed that longer telomeres were associated with higher survival in young individuals (Boonekamp *et al*. 2013). In animals, the general pattern is a positive relationship between telomere length and survival (Wilbourn *et al.* 2018). Also, early-life telomere length changes have been been found to predict lifespan in birds; for example, in captive zebra finches (*Taeniopygia guttata*, Heidinger *et al.* 2012) and wild alpine swifts (*Tachymarptis melba*, Bize *et al*. 2009). In some studies, early-life telomere length was a better predictor of survival than telomere length later in life (Ashgar *et al*. 2015).

However, not all studies find an association between survival and telomere length (Beaulieu *et al.* 2011; Sudyka *et al.* 2014). In addition, multiple studies have highlighted that telomere length may not be predictive of survival, but that the rate of attrition is (Wood & Young 2019). Even though the link between telomere length and survival is not always consistent, overall, there is a relationship between a higher mortality risk and shorter telomeres, at least in birds (meta-analysis of Wilbourn *et al*. 2018).

The differences in conclusions among studies may be due to a multitude of reasons: different methods of measuring telomeres (TRF or qPCR), leading to differences in accuracy and throughput, differences in life-history of the species studied and the durations of the studies, differences in the ages of the animals studied (*e.g.* just young or old instead of both), and potential sex differences in mortality being ignored (Wilbourn *et al.* 2018).

1.8 Heritability and parental effects

If long telomeres are beneficial to an individual, then one would expect natural selection to favour long telomeres (Belmaker *et al*. 2019). However, traits need to be heritable if they are to evolve. The heritability of telomeres has been intensively studied in eukaryotes and vertebrates, including humans, with heritability estimates (h^2) varying greatly from 0 to about 1 (Broer *et al*. 2013; Ashgar *et al* 2015; Becker *et al*. 2015; Dugdale & Richardson 2017; van Lieshout *et al*. 2019). Many heritability estimates are high, which can be due to the use of parent-offspring regressions to estimate heritability, which do not separate genetic from environmental effects (Dugdale and Richardson 2018; van Lieshout *et al*. 2020). An alternative is the use of a so called 'Animal model', which uses a pedigree and can portion phenotypic variance into genetic and environmental components (Dugdale & Richardson 2018).

Aside from genetic and environmental effects, telomere length may also be passed on in a transgenerational way. As both parents pass on telomeres to the offspring, a difference in telomere length between two chromosomes can exist. This can lead to different telomere restoration and maintenance. These mechanisms can either compensate fully for any initial differences in telomere length in the zygote, called a telomeric reset (Vizlin-Hodzic *et al*. 2009). However, if such a reset occurs, no link should exist between parental telomere length and offspring telomere lengths. Alternatively, a telomeric reset could not occur and then parental telomere lengths could be correlated to offspring telomere length. Such an epigenetic inheritance is shown in some studies

that link parental age to offspring telomere length (Criscuolo *et al.* 2017; Noguera *et al*. 2018; Bouwhuis *et a*l. 2018; Bauch *et a*l. 2019).

However, results from studies studying parental age effects on offspring telomere length have different conclusions, ranging from positive to negative parental age effects (Bauch *et al*. 2019). The variance in conclusions may be due to the use of cross-sectional studies, which assess what age-related changes in telomeres occur at the population level. Age-related changes in telomere length within-individuals cannot be assessed. As the effects at the population level (between-individuals) can differ from the within-individual age effects, discerning these two effects is vital. Only one study to date investigated the relationship between offspring telomere length and parental age within individual parents. This study in jackdaws concluded that offspring telomere length decreased within individual father age. No effect was detected within mothers (Bauch *et a*l. 2019). Seeing that we have a long-term dataset with multiple measurements of the same individual, our house sparrow population would provide valuable information about any age-related correlations between parent and offspring telomere length. Gaining insight in parental age correlations to offspring telomere length is important due to the effect such a link may have on the evolution of senescence. For example, if parental age has a negative association with offspring telomere length, and telomere length affects fitness, selection may favour young individuals to reproduce, at the cost of future reproduction. Such a link could influence the age structure of populations.

1.9 Questioning causal involvement

There are three indications that telomere lengths may have a causal effect on age and survival (Young 2018). First, several studies have shown that species with shorter telomeres tend to have shorter lifespans and individuals with shorter telomeres tend to have lower survival (Wilbourn *et al*. 2018; Tricola *et al.* 2018). Second, short telomeres have been associated with higher risks of some diseases in humans, such as gastric cancer and diabetes (Hermann *et al*. 2018; Smith *et al.* 2019). Third, when telomerase affects telomere length, and in experiments in mice where telomerase is either overexpressed or knocked-out, lifespans are affected positively and negatively, respectively (Criscuolo *et al.* 2018). However, these observations do not provide conclusive evidence that telomeres play a causal role in aging. Telomeres could instead be a biomarker of accumulated damage to other (cell) structures that cause aging (Young 2018). The finding that telomere attrition, instead of telomere length, predicts survival in some species (i.e., Boonekamp *et al.* 2013), is an indication that telomeres may indeed be reflecting accumulated damage to other biological structures that cause aging (Young 2018).

Moreover, if telomeres are causally involved with aging, two predictions follow: 1. If telomere length determines mortality at older ages, then it is expected that telomere length predicts remaining lifespan more closely at older ages than at younger ages (Simons 2015). 2. If telomeres shorten until they drop below a critical length, then individuals with the shortest telomeres would die off, leaving less variance of telomere length in the population as age increases (Simons 2015). However, in contrast the correlation between telomere length and mortality appears to decrease with age (Boonekamp *et al.* 2013), and simulated data as well as a meta-analysis in humans showed that the variance in telomere length did not decrease with age (Simons 2015).

Another reason to doubt the causal involvement of telomeres in survival is the question of why selection has not favoured individuals with longer telomeres (Young 2018). The most common argument is that trade-offs make such selection impossible or at least not beneficial, due to the cost it entails to, for example, other systems. However, that individuals of the same age and species still show high telomere length variation, and often have some heritable component (Dugdale *et al.* 2018), shows that selection could potentially act on telomere length (Young 2018). Furthermore, there are many potential mechanisms that could drive the selection, such as favouring individuals with better telomere maintenance, longer initial telomere lengths or even evolving an alternative to telomeres, as seen in plants and some animals (Blackburn 2001).

In sum, telomeres may not be the underlying cause of mortality, but may instead reflect other processes that lead to death or disease. However, the study of telomeres would not become less interesting should a causal relationship be absent. After all, as a potential biomarker of age, quality and other aspects of life, telomeres still offer a valuable tool with which to measure or predict life-history components.

1.10 Thesis outline

Chapter 2 investigates a methodological issue regarding sample storage I discovered after our telomeres were measured on the qPCR. To do so, I conducted an experiment where I re-extracted the DNA and re-measured the telomere length of 80 randomly selected samples to compare the telomere length of these samples at different storage times. After I investigated potential effects of blood sample storage on telomere length, I continued to study the biological shortening of telomeres with both within and between individual age, which will be presented in Chapter 3. In this chapter I also tested if telomere length was associated with survival and lifespan, and assessed if selective disappearance of individuals with short telomeres occurs in our house sparrow population.

In Chapter 4, I use our longitudinal house sparrow data to disentangle population level parental age effects from within-individual parental age effects. Moreover, I use our cross-fostering experiment to also disentangle any biological parental effects from social parental effects. To date, only one other study in parental age effects on offspring telomere length has included both longitudinal and cross-fostering data, but only across a few years (Belmaker *et al.* 2019).

In Chapter 5, I focused on partitioning the phenotypic variance of telomere length into different components, such as genetic, environmental, paternal and maternal. There are only a few studies to date that have estimated the heritability of telomere length in a wild population. Most of these studies employ parent–offspring regressions to assess genetic contributions to telomere length in offspring. Only a few studies use animal models, which more effectively allow genetic and environmental contributions to telomere length to be separated. As the Lundy house sparrow has one of the most complete pedigrees constructed for a natural population, it is highly suitable for applying the animal model.

In Chapter 6 I provide a short summary of my thesis, and make a few general conclusions regarding my findings, as well as pointers to the future of telomeres.

1.11 Contributions made to this thesis

Blood samples were collected on Lundy over a range of years by many PhD students, master students and field assistants. In the laboratory, DNA was extracted, and telomeres were measured by Nathalie Dos Remedios and Marie-Elena Mannarelli. Telomere ratios were calculated using an R script provided by Mirre Simons.

I confirm that this Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously been presented for an award at this, or any other, university.

2. Blood sample storage in ethanol affects telomere length estimation by qPCR

2.1 Summary

The role of telomeres as biomarkers of health and aging has received increasing interest in the past years. Many of the resulting studies, first *in vitro* and studied in humans, but later also assessing telomere dynamics across the lifespan of wild animal populations, collect blood samples across many years. Consequently, the blood samples may be stored for different storage durations before DNA is extracted and telomeres length measured.

The varying sample storage times may affect telomere length, as previous studies have indicated. However, few studies have designed an experiment to test specifically if storage time of blood samples in ethanol is affecting telomere length when measured by quantitative PCR (qPCR). Moreover, the assessed storage time in previous studies has rarely surpassed one year, which may not be representative of the storage time in most longitudinal studies.

We used blood samples that were stored in ethanol for between one and ten years at room temperature to assess the effect of sample storage time until DNA extraction and qPCR measurement on telomere length. We detected a negative effect on telomere length measurements of the time stored as a blood sample in ethanol at room temperature until DNA extraction. We found no effect of DNA storage time on qPCR measurement. Our findings highlight the importance of extracting DNA from blood samples soon after blood sample collection, freezing samples, or assessing blood sample storage time before analysis of biologically relevant questions, even when the same sampling storage protocol is used for all samples within a study.

2.2 Introduction

Telomeres are repetitive DNA sequences ($[TTAGGG]_n$) located at the ends of chromosomes (Meyne *et al.* 1989; Blackburn 1991). Past studies have indicated that telomeres may play a fundamental role in the protection of chromosomal DNA, as well as a role in the regulation of cellular senescence (Armanios & Blackburn 2012; Nussey *et al*. 2014). Moreover, telomeres appear to shorten with age and other metabolically costly life experiences, and, as such, may reflect the (cumulative) effects of stresses from the environment as well as predicting susceptibility to disease (Blackburn *et al*. 2015; De Meyer *et al*. 2018; Willis *et al*., 2018). Consequently, telomere length may predict survival (Bize *et*

al. 2009; Heidinger *et al*. 2012; Nussey *et al*. 2014; but see Boonekamp *et al*. 2014). Because telomeres may predict longevity, telomeres may explain variation in life-history traits and also provide insight into the evolution of life-history strategies (Haussmann 2008).

As telomeres may reflect variation in life-history traits, studies in evolutionary ecology have tried to explain the variation in telomere length (Nakagawa *et al*. 2004; Haussmann & Marchetto 2010; Horn *et al*. 2010; Monaghan 2010). In order to study telomere dynamics, tissue samples – usually blood – are collected and stored until the DNA is extracted for telomere measurement. There are a multitude of available techniques to store samples, extract DNA and measure telomere length, each usually requiring high competence levels in order to result in accurate estimates of TL. Several studies have now indicated some important concerns with the methods applied to extract DNA and measure telomere length (Horn *et al*. 2010; Haussmann *et al*. 2011; Nussey *et al.* 2014). However, the conditions of storage of (blood) samples, and their duration, have undergone less scrutiny.

Degradation of DNA over time might especially be a potential issue when measuring telomeres, as telomeres are located at the ends of chromosomes, which are especially susceptible to degradation (Wang *et al*. 2010). The degradation of telomeres may be an issue when measuring telomeres with the quantitative Polymerase Chain Reaction (or qPCR). qPCR utilises a reference gene, which is measured at the same time as the telomeric DNA, to calculate the ratio of telomeric DNA to the DNA of the reference gene (Cawthon 2009). Due to the location of telomeric DNA at the end of chromosomes, it may degrade more rapidly than the reference gene DNA, which would affect the resulting ratio. The effect on the ratio may not only be negative, as would intuitively be expected. The quadruplex structures at the 3' end of telomeres can become more accessible to qPCR over time, which may result in a in a higher telomere measurement, and consequently a higher *T/S* ratio (Zanet *et al*. 2013).

Different storage methods are available in which to store blood samples, such as whole-blood storage in ethanol, in ethylenediaminetetra acetic acid (EDTA), in acid citrate dextrose (ACD), or as a dried blood sample on Flinders Technology Associates (FTA) cards. Whether telomeric DNA is affected by storage may depend on the storage method used (Tolios *et al*. 2015). In a study by Reichert *et al*. (2017), blood samples on FTA cards were compared to those stored at -80°C, and it was concluded that samples from FTA cards had significantly smaller telomere : control gene ratios. Dagnall *et al*. (2017) concluded that the DNA concentration of the sample and the temperature affected telomere length negatively. Eastwood *et al.* (2018) collected and stored samples in ethanol

at 4°C for 7 months. This method was compared to two other storage methods, in Longmire's and Queen's lysis buffers, also stored at 4°C for 7 months (Eastwood *et al.* 2018). The study concluded that, at least for their study system, storage in ethanol yields good results (at least when paired with optimized assays and extraction protocols) because telomere measurements were higher when the blood was stored in ethanol, whilst Longmire's and Queen's lysis produced lower telomere estimates. The previous studies and the varying effects of storage methods on telomere length show that scrutiny is needed when determining the storage method for a study.

The previous studies focused on DNA extraction or storage methods and very few studies have addressed the effects of storing storage time on telomere length, while in blood samples the length of time before a (blood) sample's DNA is extracted or telomere length is analysed can also potentially affect telomere length. There are only a few studies that included effects of storage time on telomeric DNA in their research. Zanet *et al*. (2013), stored whole blood samples in EDTA or ACD at room temperature for 9 days. The telomeric DNA appeared stable for the first 4 days, but showed degradation after that. A study in the Seychelles warbler, *Acrocephalus sechellensis*, where samples were collected and stored in ethanol at room temperature, reports finding no relationship between sample storage time and telomere length (Spurgin *et al*. 2017). However, there is no mention of for how long the samples were stored before DNA extraction and analysis.

If samples can be affected by storage length, both cross-sectional and longitudinal datasets are at risk of basing conclusions on methodological (storage) effects instead of biological effects, as any potential biological effects may be confounded with methodological storage time effects. This risk is increased in biological studies because, oftentimes blood samples may be stored over a long period of time as samples are collected in the field and DNA extraction and telomere measurement may not be readily available. For some of the samples, the telomere length is not measured until many years after sample collection (Lin *et al.* 2019). Depending on laboratory set-up, some samples may be measured sooner than others, resulting in different storage times. These different storage times may influence DNA quality and quantity and consequently telomere length results and conclusions (Nakagawa *et al*. 2004; Haussmann *et al*. 2011; Smith *et al*. 2011; Cunningham *et al*. 2013; Nussey *et al.* 2014).

We originally aimed to analyse telomere dynamics in our long-term dataset of house sparrows, P*asser domesticus*, in which samples were collected in the field and then stored in ethanol at room temperature. Initial investigation of the data indicated a decline of telomere length with sample

storage time. Consequently, we were interested in how long-term storage time (up to 10 years) affects telomere length estimates in our dataset. To test if storage time affects telomere length, we randomly selected 80 samples that were previously measured from our long-term dataset. We then re-extracted the DNA and re-measured telomere length in the selected samples by qPCR to assess effects of storage time on telomere length. This experiment allowed for repeated measures of the same sample at different sample storage ages, which allowed the detection of any potential within-sample effects of storage time on telomere length. This study is the first to test whether telomere length is affected by storage time across multiple years, and where the sample storage time is divided into pre-extraction and post-extraction storage.

2.3 Methods

We selected 80 samples from our long-term dataset. Our long-term dataset consisted of 2,676 samples which were collected from house sparrows on Lundy island (51.11 N, 4.40 W) in the years 2000 to 2015. House sparrows are small passerines (Summer-Smith 1963). All individual sparrows were uniquely colour ringed and provided with a metal numbered ring from the British Trust for Ornithology. Adults were caught in both summer (April–September) and winter (November–March) using mist-nets or trapping within nestboxes and funnel traps.

2.3.1 Sample collection

The samples from fledged individuals (individuals that had left the nest) were collected and then stored in 1 ml of *ca* 100% ethanol at room temperature until DNA was extracted.

2.3.2 DNA extraction

DNA was extracted from blood samples prior to analysis and subsequently stored at -20°C in low TE pH 7.5. Prior to qPCR, DNA was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Fisher) and normalised to 20–30 ng/ul. DNA purity was checked on the Nanodrop, using the Nucleic Acid ratios 280/260.

For qPCR, a multiplex protocol was implemented following Cawthon 2009. DNA was measured using a StepOnePlus from Applied Biosystems or Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific), measuring telomere (T) and single copy gene (S) signals in the same reaction. The multiplex method is expected to be more accurate because any differences in the amount of DNA

that is pipetted no longer contributes to variation in the T/S ratio, because both *T* and *S* are measured from the same sample and within the same reaction (Cawthon 2009).

2.3.3 Telomere qPCR measurement

For our long-term dataset, we were aware that the collection of samples over a range of years may lead to samples being clustered by, for example, year or cohort (van Lieshout *et al.* 2020). To avoid confounding the effects of cohort with qPCR plate, each cohort of samples was randomly divided into three subsets for qPCR (quantitative polymerase chain reaction) analysis following advice from (van Lieshout *et al.* 2020). qPCR telomere measurements were undertaken in 2013–2018 (except for 2017), with several plates being analysed each year. Each plate contained a random subset of samples from each of three different collection years. This meant that, for example, one-third of a plate would contain a random subset of samples collected in Year 1, one third from Year 2 and one third from Year 3; locations on the plate were randomised.

Two sets of primer pairs were included in each reaction: telg (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3') and telc (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), targeting telomeric sequence. The difference between these two primers is that telg can prime DNA from cycle one, while telc can prime DNA from cycle two onwards. In cycle one telg and telc primer can both hybridize to native telomere sequences, but only telg can prime DNA synthesis. The telc cannot prime DNA synthesis, due to its 3' terminal mismatch. In Cycle two, telc can hybridize along telg primer extension products that were synthesized in Cycle 1, but are limited in priming DNA synthesis. In the following cycles telc can use telg extension products as a template to prime DNA (Cawthon 2009).

We used GAPDH-F (5'-CGGCGGCGGGCGGCGCGGGCTGGGCGG-AGCCAGCCAAGTACGATGACAT-3') and GAPDH-R (5'-GCCCGGCCCGCCGCGCCCGTCCCGCCG-CCATCAGCAGCAGCCTTCA-3') to amplify *GAPDH* (*Glyceraldehyde 3-phosphate dehydrogenase*), an autosomal, single copy gene, as a reference. These primers are designed to place GC-clamps on both ends of *GAPDH*, which raises its melting temperature. Because the telomeric DNA is more abundant than the *GAPDH* DNA, telomeric DNA is picked up at earlier qPCR cycles than the *GAPDH* DNA (Cawthon 2009).

The fluorescence generated by SYBR Green is indicative of the amount of PCR product in the reaction, if the reaction is in the exponential phase of the amplification curve, and provided that amplification is comparable and sufficient in all samples. The threshold was set within the log-linear

phase for all samples, well above background fluorescence (visual inspection). This enabled the calculation of the actual amount of initial molecules, since the fluorescence intensity is proportional to the amount of PCR product in the exponential phase.

Samples were run in duplicate on each qPCR plate. qPCRs were conducted in 20-µl reactions, including 1.5 µl normalised DNA, 10 µl SYBR Select Master Mix (Applied Biosystems) and each of the four primers at 0.9 µM. qPCR amplification was conducted on a Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific), as follows: 50°C for 2 mins; 95°C for 2 mins; 2 cycles of 94°C for 15s and 49°C for 15s; 40 cycles of 94°C for 15s, 62°C for 10s, 74°C for 15s (with signal acquisition), 84°C for 10s and 86°C for 15s (with signal acquisition). Note that the 74°C measure provided the Ct values (the value at which the fluorescence of the target DNA rises above background fluorescence and can thus be measured) for amplification of telomere template, whereas the 86°C measure provided the Ct values for amplification of *GAPDH* template, while the telomere product is melted. Five plates were analysed on an earlier model qPCR machine (StepOnePlus from Applied Biosystems) than the other 77 plates, using the same cycle protocol as Quantstudio 12K Flex Real-Time PCR System. To ensure that both machines provided comparable outputs, the intercept of the Ct values of the standardised curves (from the standard concentrations) from all plates from both machines were compared for both telomere DNA and *GAPDH*. Neither the telomere DNA nor the GAPDH Ct intercept values were normally distributed, hence a Wilcoxon test was done to test if the Ct intercepts differed significantly between machines; there was no difference (Telomere DNA: w = 158, p = 0.51; GAPDH: w = 215, p = 0.67, N = 82 plates).

On every qPCR plate, a standard reference sample was used to create standard curves. This reference sample was a blood sample from one of six individual birds that were selected due to the large quantity of collected blood. The DNA from each of the six reference samples was extracted at the start of the study and subsequently stored in multiple aliquots at -20°C in low TE pH 7.5. Each aliquot was used for multiple plates. This meant that one aliquot of the reference samples underwent multiple defrost cycles before a new aliquot was used.

The reference samples were run in duplicate at concentrations of 80, 20, 5, 1.25 and 0.3125 ng/µl, as well as two non-template control wells to provide the standard curves of both telomeric as *GAPDH*. The standard curves of each plate were visually assessed for outliers and a maximum of 2 outliers were removed if the amplification efficiencies of *GAPDH* or telomere DNA improved. This resulted in 15 plates having increased amplification efficiencies, improving average efficiency of all 82 plates of

the reference gene from 93.5% (range 73–119%) to 94.5% (range 76% - 119%) end efficiency of telomere DNA from 89.5% (range 71%- 109%) to 90.7% (range 71% - 109%).

Samples for which Ct > 25, indicating very low amplification success, for either *GAPDH* or telomeres were excluded from analysis, as they were deemed unreliable. As a proxy for telomere length, we calculated the ratio of telomere signal (*T*) to *GAPDH* signal (*S*). Ct values for the telomere DNA and GAPDH signal per well were evaluated using the standard curve for each plate. *T* and *S* were calculated from the formula:

Standardised signal = 10 ^ ((*Ct* value of sample – Intercept of standard curve) / log coefficient of standard curve)

We then calculated the *T*/*S* ratio for each sample.

As samples were analysed in duplicate in two separate but adjacent wells on each plate, and thus are expected to have similar *T/S* measurements, outliers were detected by calculating the relative difference between duplicates:

((*T/S* ratio replicate 1 – *T/S* ratio replicate 2) / (*T/S* ratio replicate 1 + *T/S* ratio replicate 2)) /2

Replicates that had a relative *T/S* ratio difference of more than 0.2 were re-analysed by qPCR. If the relative difference was still more than 0.2, the samples were omitted from analysis. Some samples were measured in duplicate multiple times (on different plates) without exceeding the 0.2 difference. If that were the case, samples with the least relative difference were selected for analysis.

2.3.4 Experiment

We randomly selected 80 blood samples from our long-term dataset using R (version 3.5.0; R Core Team 2018). Degradation may occur during the period stored as a blood sample (hereafter referred to as blood storage time) or after the DNA has been extracted and stored at -20 °C (hereafter referred to as DNA storage time). To separate these two possible effects, we re-extracted the DNA and repeated the qPCR telomere measurements on both the 80 new and 80 old DNA extractions in 2018. This created a qPCR dataset that included repeated measures on the same blood samples that

incorporated large variation in both blood sample storage time and DNA storage time. The protocol for extracting DNA and measuring the samples on the qPCR are equal to the protocols used for our long-term dataset (2.2.3).

As the 80 randomly selected samples were part of the larger long-term dataset, of which the samples had their DNA originally extracted anywhere between 2006 and 2016, and their telomeres measured between 2013 and 2018 (situation A in Figure 2.1). The 80 selected samples had their DNA extracted again and were re-measured on the qPCR machine in 2018 (situation C in Figure 2.1). This led to a longer blood-storage time and a shorter DNA storage time when compared to the measurement from our long-term dataset (situation A), because the blood samples were stored for a longer period but DNA extractions were measured immediately after extraction. We also re-measured the old DNA extractions (as stored previously from our long-term dataset) in 2018, leading to a longer DNA storage time and no change in blood storage time compared to the initial measurement (situation A in Figure 2.1).

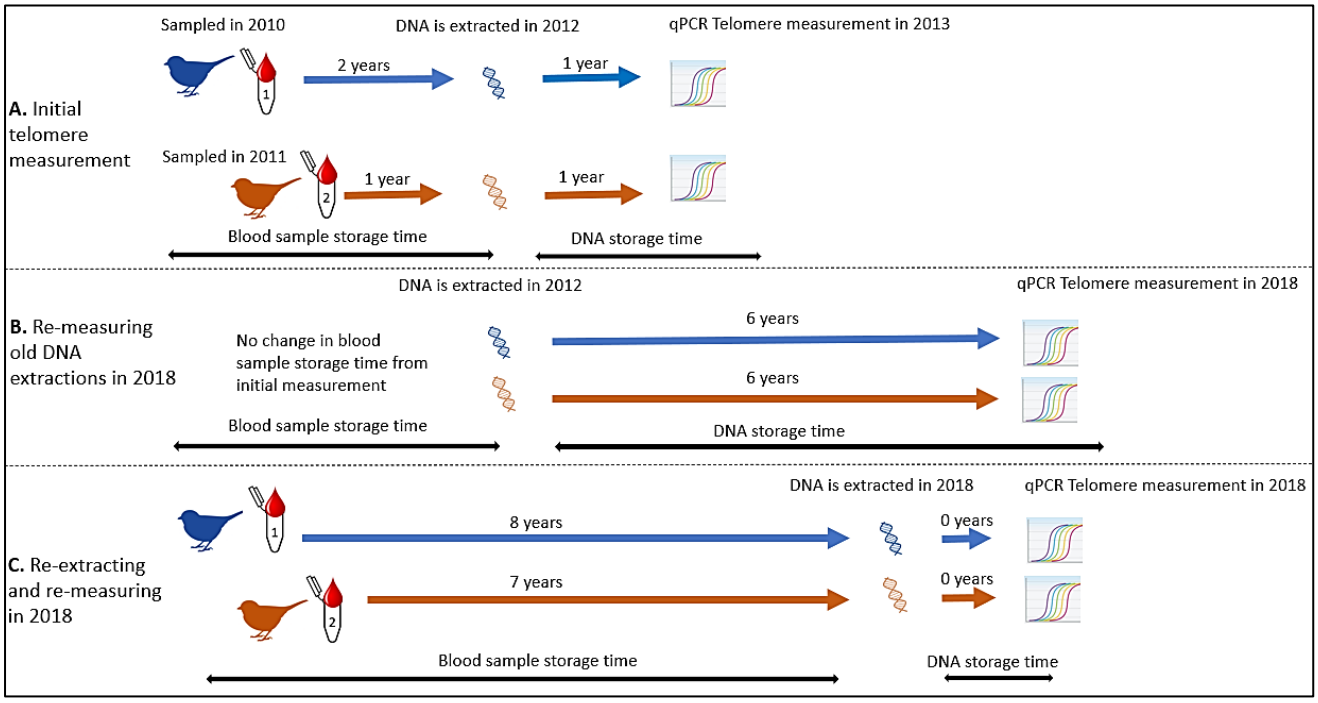


Figure 2.1. Two examples showing the differences in blood storage time and DNA storage time compared in our experiment. The examples show two blood samples taken in 2010 and 2011. A. Our initial measurement from our long-term dataset. The samples are taken from an individual in 2010, and another individual in 2011. DNA for both birds is extracted one and again two years later, while the qPCR measurement was completed one year later for both samples. B. In 2018 we remeasured the old DNA extractions, as stored at -20°C from our long-term database. This is 6 years later, as the DNA was stored in 2012 for both individuals. The blood sample storage time does not change, as only the extracted DNA was re-measured. C. We re-extract the DNA in 2018 from blood samples collected in 2010 and 2011 to remeasure the telomeres in 2018. The time stored as a blood sample is now 8 and 7 years, respectively, for our two examples. The DNA storage time is 0 because the DNA is measured immediately after DNA extraction.

2.3.5 Statistical analysis

All statistical analyses were conducted in the R environment (version 3.5.0; R Core Team 2018) using the 'lme4' package (version 1.1 – 17). The statistical analyses were performed for the 80 samples and their measurements from the three time points at which they were (re)measured (Situation A, B and C in Figure 2.1). The natural log was taken of the *T/S* ratio for all models (hereafter referred to as RTL).

Because it is unknown when any potential degradation occurs – during the time stored as blood sample or as DNA extraction – we tested separately for the effect of blood storage time and DNA storage time. The effect of blood age and DNA age on telomere length were tested in a Linear Mixed Model (LMM). In this model, RTL was used as a response variable. For both blood and DNA age (in years) we applied Van de Pol & Wright's (2009) method, calculating the mean and centred storage times for the sample to allow for assessment of both between- and within-sample storage time effects on telomere length.

In a separate analysis we tested whether blood storage time (in years) had a linear or quadratic relationship with telomere length. We used an LMM, where RTL was added as the response variable, while both the linear and squared effects of blood storage time were added as fixed effects. Due to the repeated measurement of the same samples, and samples being analysed on the same or different qPCR plates, sample identity and qPCR plate identity were added to all models as random intercepts.

Because telomere length is expressed as a ratio of *T* and *S* values, an association between storage time and telomere length can be caused by a change in length of either telomeric DNA, reference gene DNA or both. We therefore tested if the *Ct* values of both telomeres and the reference gene (*GAPDH*) were related to storage time in four separate linear mixed models. In the first two models either the *Ct* value of telomeric DNA or *GAPDH* was set as the response variable, and the time stored as a blood sample as both linear and quadratic factor was added as a fixed covariate. We also added the sample identity and qPCR plate identity as random intercepts to avoid pseudo replication. For the third and fourth model, we used models one and two, but replaced the time stored as a blood sample with the time stored as a DNA sample.

Our 80 samples had, on average, an initial blood storage time of 2.8 years (*SD* 3.8) and a DNA storage time of 1.2 years (*SD* 1.2). When the old DNA extractions were remeasured, the mean blood

storage time was 3.3 years (*SD* 4.1) and the DNA storage time was 4.2 years (*SD* 1.2). The reason the blood storage time was not the same as the initial blood age was that some samples did not meet our criteria (such as a minimum of 0.2 relative difference between duplicates of the same sample on a plate or *Ct* > 25 for either *GAPDH* or telomeres), and were thus excluded, changing the average blood age. When the DNA was re-extracted and re-measured, the mean blood age was 7.3 years (*SD* 4.1) and the DNA age was 0.

2.3.6 Repeatability of telomere lengths

We were interested in the repeatability of RTL estimates to assess the reliability of our qPCR measurements. We analysed RTL sample repeatability using the rptR package (Stoffel *et al*. 2017). rptR uses a mixed linear model framework, where the repeatability is calculated as the variance among the means of groups (in our case blood samples) over the sum of group-level and data-level (residual) variance. In this model we used RTL as the dependent variable and sample identity as a random effect. The repeatability of duplicates of the same sample (samples are always measured in two separate wells on a single qPCR plate) was 0.98 (se = 0.004, *N* = 145 samples), meaning that 98% of the variance observed in RTL can be explained by within-sample consistency.

To assess the repeatability of the 80 samples that had their DNA re-extracted and telomeres re-measured on the qPCR machine, we selected all samples that had two or more sample measurements. All samples were re-measured, but some measurements had to be excluded due to the duplicates of one sample on a plate having a larger relative difference than 0.2 or having *Ct* values of GAPDH or telomeres > 25. The repeatability of RTL of samples that were either re-analysed and/or re-extracted (and thus had varying blood and DNA storage times) was 0.49 (rpt, se = 0.07; *N* = 58 samples). Adding the qPCR plate as a random effect to the model to assess repeatability of samples lowered the repeatability to 0.37 (rpt, se = 0.08, *N* = 58 samples).

2.4 Results

Using a model that included both mean and centred blood and DNA storage times, RTL was only associated with the blood storage time ($\beta_{\text{mean blood storage time}}$ = -0.05, se = 0.01, t = -6.9, df = 58.3, $p < 0.001$ and $\beta_{\text{centred blood storage time}}$ = -0.08, se = 0.03, t = -3.1, df = 35.8, $p = 0.004$). The DNA storage time had no effect on relative telomere length ($\beta_{\text{mean DNA storage time}}$ = 0.02, se = 0.03, t = 0.5, df = 77.8, $p = 0.61$ and $\beta_{\text{centred DNA storage time}}$ = -0.03, se = 0.03, t = -0.98, df = 40.3, $p = 0.33$, Table 2.1).

Upon further investigation of the relationship between telomere length and blood storage time, we found that RTL was negatively associated with blood storage time ($\beta_{\text{blood storage time linear}}$ = -0.10, se = 0.01, t = -6.90, df = 173.7, $p < 0.001$ see Table 2.2), and that this effect showed a quadratic relationship ($\beta_{\text{blood storage time}^2}$ = 0.004, se = 0.001 t = 3.41, df = 173.5, $p < 0.001$). See Table 2.2 and Figure 2.1 for more details.

Table 2.1. The linear mixed model results, testing for mean and centred blood storage and DNA storage time effects on log RTL.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.327	0.073	4.449	99.4	**<0.001**
Mean blood storage time	-0.053	0.008	-6.904	58.3	**<0.001**
Centred blood storage time	-0.084	0.027	-3.077	35.8	**0.004**
Mean DNA storage time	0.016	0.032	0.507	77.8	0.614
Centred DNA storage time	-0.028	0.028	-0.978	40.3	0.334
Random effects	**Variance**	**Stan. dev.**			
Blood sample identity	0.033	0.180			
qPCR plate identity	0.029#4	0.156			
Residual	0.056	0.237			

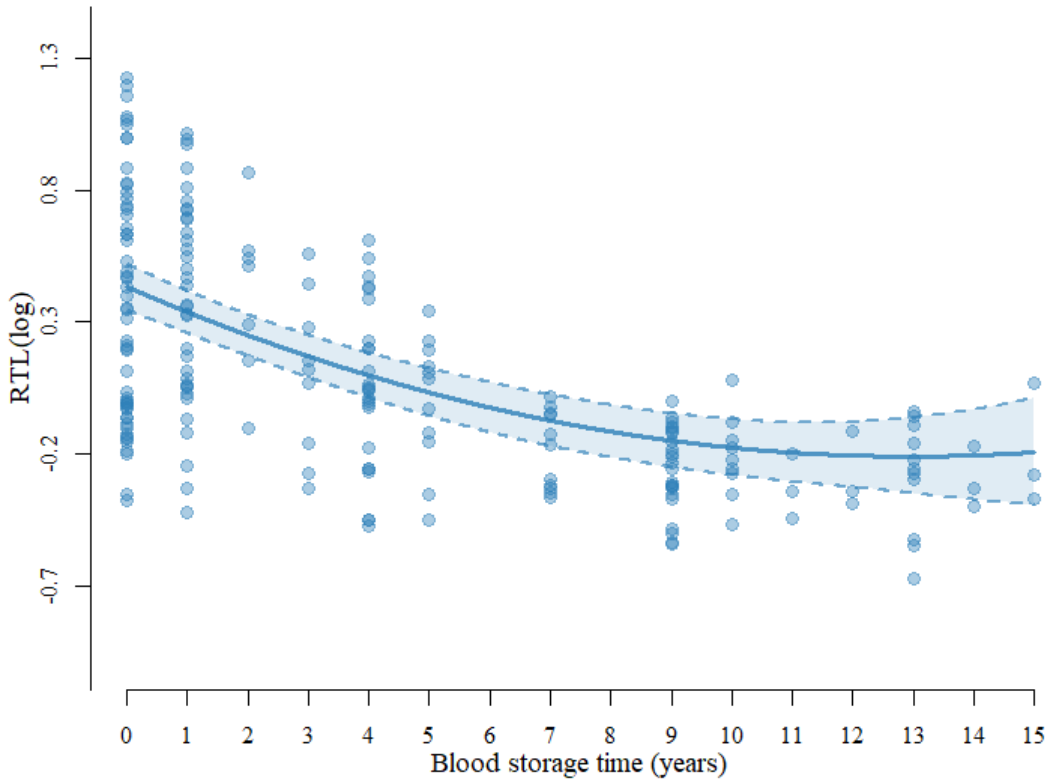


Figure 2.1. Quadratic blood sample age effect on RTL. The solid line represents the model fit, the dashed line the 95% credible interval.

Table 2.2. blood sample storage effects on RTL, linear mixed model results.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.434	0.044	9.812	101.3	**<0.001**
Linear blood storage time	-0.099	0.014	-6.900	173.7	**<0.001**
Quadratic blood storage time	0.004	0.001	3.417	173.5	**<0.001**
Random effects	**Variance**	**Stan. dev.**			
Blood sample identity	0.031	0.177			
qPCR plate identity	0.021	0.146			
Residual	0.053	0.230			

We found no association between telomere *Ct* values and linear nor quadratic blood sample storage time ($\beta_{\text{blood storage time}}$ = 0.00, se = 0.03, t = 0.06, df = 158.2, *p* = 0.95 and $\beta_{\text{blood storage time}^2}$ = -0.00, se = 0.00, t = -0.19, df = 159.4 *p* = 0.85, respectively). We did find a negative association between *GAPDH Ct* values and linear blood storage time ($\beta_{\text{blood storage time}}$ = -0.12, se = 0.04, t = -3.13, df = 178.9, *p* = 0.002). There was no relationship between *GAPDH Ct* values and quadratic blood sample storage time ($\beta_{\text{blood storage time}^2}$ = 0.00, se = 0.00, t = 1.36, df = 176.7, *p* = 0.18, Table 2.3).

Table 2.3. Blood sample storage effects on telomere and *GAPDH Ct* values, linear mixed model results from two models.

	Ct telomere					*Ct GAPDH*				
Fixed effects	**Estimate**	**se**	**t**	**df**	***p***	**Estimate**	**se**	**t**	**df**	***p***
Intercept	18.882	0.150	125.284	57.7	**<0.001**	21.079	0.143	147.531	68.0	**<0.001**
Linear blood storage time	0.002	0.034	0.061	158.2	0.951	-0.118	0.038	-3.131	178.9	**0.002**
Quadratic Blood storage time	-0.000	0.003	-0.193	159.4	0.847	0.004	0.003	1.357	176.7	0.176
Random effects	**Variance**	**Stan. dev.**				**Variance**	**Stan. dev.**			
Blood sample identity	0.083	0.289				0.061	0.247			
qPCR plate identity	0.736	0.858				0.565	0.752			
Residual	0.262	0.512				0.359	0.600			

For DNA storage, there were no associations between the Ct values of GAPDH and linear DNA storage time ($\beta_{\text{DNA storage time}}$ = 0.08, se = 0.05, t = 1.67, df = 164.0, *p* = 0.10). There was also no correlation between the Ct values of GAPDH and quadratic DNA storage time ($\beta_{\text{DNA storage time}^2}$ = 0.01, se = 0.01, t = 0.88, df = 172.2, *p* = 0.38). There were no associations between the Ct values of telomeres and linear nor quadratic DNA storage time ($\beta_{\text{DNA storage time}}$ = 0.01, se = 0.04, t = 0.20, df = 150.9, *p* = 0.84 and ($\beta_{\text{DNA storage time}^2}$ = 0.01, se = 0.01, t = 0.89, df = 160.1, *p* = 0.37, respectively). See Table 2.4 for linear mixed model output.

Table 2.4. DNA sample storage effects on telomere and *GAPDH Ct* values, linear mixed model results from two models.

	Ct telomere					*Ct GAPDH*				
Fixed effects	**Estimate**	**se**	**t**	**df**	***p***	**Estimate**	**se**	**t**	**df**	***p***
Intercept	18.790	0.145	129.358	51.3	**<0.001**	20.690	0.144	144.101	62.4	**<0.001**
Linear DNA storage time	0.008	0.040	0.204	150.9	0.838	0.075	0.046	1.669	164.0	0.097
Quadratic DNA storage time	0.005	0.006	0.891	160.1	0.374	0.006	0.007	0.877	172.2	0.382
Random effects	**Variance**	**Stan. dev.**				**Variance**	**Stan. dev.**			
Blood sample identity	0.074	0.271				0.040	0.201			
qPCR plate identity	0.747	0.864				0.666	0.816			
Residual	0.260	0.510				0.370	0.608			

2.5 Discussion

To our knowledge, this is the first systematic examination of long-term blood sample storage time in ethanol at room temperature, which is an often-used method of storage in field biology (Arctander 1988; Owen 2011), on telomere measurements. In our study, we found evidence that the time a sample was stored as a whole blood sample in ethanol at room temperature shortens telomeres as estimated by qPCR, and that this effect was reduced during the time in storage. We did not find any associations between telomere length and storage time for frozen, extracted DNA, suggesting that storing frozen DNA does not affect telomere length.

Collected whole blood samples can show a quick change in telomere length during storage (Zanet *et al*. 2013). The *T/S* ratio of whole blood samples that were stored in an anticoagulant (ACD or EDTA) at 4°C and room temperature remained stable for the first 4 days, but showed an increase in *T/S* ratio after 4 days, up to a 34% increase after 9 days (Zanet *et al*. 2013). Such an increase in telomere length with storage time can occur when the telomere amplification in the qPCR increases relative to the reference gene because, due to degradation, the quadruplex structures at the 3' end of telomeric DNA become more accessible for primers during the qPCR, leading to more amplification of telomeric DNA (Zanet *et al*. 2013).

Why telomere lengths appear to shorten during whole-blood storage in our study is unclear, and we can only speculate as to the cause. In comparison, blood samples stored on FTA cards show signs of DNA fragmentation because PCR reaction efficiency was lower, and gels showed the DNA was more fragmented (Reichert *et al*. 2017). However, in contrast, Rosero-Bixby *et al*. (2019) found no sign of DNA fragmentation in extracted DNA samples that were stored at 4°C for multiple years, then analysed on agarose gel. We can only speculate that whole blood samples stored in ethanol also undergo DNA fragmentation. DNA fragmentation can potentially occur due to the non-sterile conditions in which samples are collected and stored. Due to the lack of completely sterile storage

conditions, *T/S* ratios can be affected by, for example, bacterial and fungal nucleases affecting telomere lengths, or oxidation of DNA affecting the PCR reactions of *T* and *S* differently (Rosero-Bixby *et al*. 2019). We statistically tested whether Ct values for telomeres, our reference gene (*GAPDH)* or both were affected by the time period for which a sample was stored, and found that the *Ct* values of telomeres remained stable through both blood sample and DNA extraction storage, while the *Ct* values of *GAPDH* went down with blood-sample storage time. Lower *Ct* values of GAPDH indicate that more *GAPDH* DNA was detected in the qPCR, leading to higher *S* values and consequently lower *T/S* ratios because telomeres were unaffected by blood-storage time. We can only speculate why *GAPDH* was positively affected by blood sample storage time. It is possible that due to DNA fragmentation, the *GAPDH* DNA became more accessible over time for priming, resulting in more DNA being amplified in older blood samples. We did not check for DNA degradation in our dataset prior to qPCR analyses - by, for example, using agarose gel electrophoresis -, so we cannot exclude that the DNA of samples degraded over time. In contrast, we detected no correlation between DNA storage time and the Ct values of *GAPDH*. This could be the result of the different storage methods, where blood samples were stored in ethanol at room temperature, while DNA was stored at -20°C in low TE pH 7.5. Moreover, telomere measurements remained unaffected in both blood sample and DNA storage, which may be due to the small repeats of telomeric DNA, where DNA fragmentation has little impact on priming ability.

There is only one other study that specifically assessed the effects of whole-blood sample storage in ethanol on telomere length. This study from Eastwood *et al*. (2018) concluded that storage in ethanol at 4°C yielded the highest relative telomere length, when compared to storage in Longmire's and Queen's lysis buffer. It should be noted that, as shown by the increase in telomere length with storage time in Zanet *et al.'*s (2013) study, obtaining higher telomere length estimates does not necessarily equate to better estimates. However, Eastwood *et al.* (2018) also reported high precision when blood samples were stored in ethanol. Regardless, the samples in Eastwood *et al.'*s (2018) study were only stored for 7 months, and were obtained from only 26 individuals, so their conclusions may have limited predictive value. To illustrate the point that attrition of telomere length may not be detected in blood samples that were stored for a relatively short time (less than a year), we restricted our dataset only to samples that were stored as a blood sample in ethanol at room temperature for a maximum of one year. This subset contained 52 samples. Within this subset, we also did not detect a decline in *T/S* with blood sample storage time (LMM, $\beta_{\text{blood storage time}}$ = 0.02, *se* = 0.11, t = 0.19, *df* = 50.4, p = 0.95, Supporting information Table S2.1).

We did not detect any association between telomere length and time of DNA storage at -20°C. This is in agreement with another study that assessed storage-condition effects on blood samples from birds (Reichert *et al*. 2017). Reichert *et al.'*s (2017) study concluded that telomere length was highest for samples that were stored as frozen DNA extractions, when compared to whole-blood samples that were frozen or stored on FTA cards. However, their study cannot exclude the possibility that the differences stemmed from other sources, such as different DNA extraction methods, as these were not consistent in their study (Reichert *et al*. 2017). As our study has used the same extraction and qPCR measurement methods throughout, we can exclude the possible confounding effects of DNA extraction and qPCR quantification. As such, we can conclude that in our study (frozen) extracted DNA was more stable than whole-blood samples in ethanol.

In contrast, Rosero-Bixby *et al*. (2019) did find effects of DNA extraction storage on telomere length in human blood samples. This study stored DNA extractions at 4°C for up to 9 years and concluded that telomere ratios went up in the first 5 years of storage, after which the pattern reversed. The DNA extractions in this study were not frozen, which seems likely to explain why they did find an effect of DNA storage on telomere length.

The negative effect of blood sample storage time has implications for further research, as it could confound biological effects in the study species. For example, the age of individuals may be correlated with blood sample age, as samples from older individuals may have been stored for a longer time. This means that it will be hard to distinguish biological cohort effects from artefactual blood storage effects. Moreover, not only samples may be affected by storage time, but also the reference samples that are used in qPCR can be affected. For example, in our study, the DNA of all reference samples was extracted at the beginning of qPCR analyses and stored in multiple aliquots before freezing. One aliquot was used for multiple assays, and thus underwent multiple defrost cycles. We know that freeze/thaw cycles of whole blood minimally impacted qPCR T/S ratios (Zanet *et al*. 2013). However, whether frozen DNA remains unaffected by freeze/thaw cycles, especially after long periods of storage, is unknown. Reference samples being differently impacted due to a difference in how many freeze/thaw cycles an aliquot underwent, may result in a difference between the T/S ratios of samples between plates, also potentially confounding biological effects of interest.

Past studies emphasise the need for consistency in sampling protocols. Our findings add that even when the method of storage is kept constant throughout a study, there is still the possibility of an

effect of storage time on telomere length measurements, potentially confounding biological effects. We also find that, even though the telomere to reference gene ratio may remain unaffected (in our study, with regards to DNA storage time), the telomere and/or reference gene values can potentially change independently of each other with storage time. This means that no change in the *T/S* ratio does not equate to no changes in telomere and reference gene length with storage time. It is essential for studies to investigate any potential changes in either telomere or reference gene length with storage time, to validate *T/S* ratios. We also recommend that DNA should be extracted from blood samples as soon as possible after collection. If whole-blood storage is required, freezing the blood samples should minimize telomere attrition. For datasets that have stored samples without freezing, we recommend testing for storage-related effects on telomere measurements. When such effects are found, consideration should be given to controlling for storage time as a covariate in statistical models.

Supporting information S2.

We selected only samples that were stored for 0 or 1 years (as a blood sample in ethanol at room temperature) to assess if a decline in telomere length is detectable with relatively short blood sample storage times.

Table S2.1. blood sample and DNA storage effects on RTL measurements in samples stored for 0 or 1 year. Results from linear mixed model.

Fixed effects	**Estimate**	**se**	**t**	**df**	***p***
Intercept	0.395	0.084	4.720	68.0	**<0.001**
Blood storage time	-0.003	0.028	-0.110	35.4	0.913
DNA storage time	0.011	0.011	0.096	48.8	0.924
Random effects	**Variance**	**Stan. dev.**			
Blood sample identity	0.088	0.297			
qPCR plate identity	0.022	0.149			
Residual	0.062	0.249			

3. Longitudinal data reveal selective disappearance of individuals with short telomeres

3.1 Summary

Telomere length shows high variation between individuals, even between those of the same age. This variation is hypothesised to be caused by either differences in telomere length at birth or in how telomeres shortens with age. Most studies investigating telomere shortening have been cross-sectional. More recently, however, longitudinal studies have shown that telomeres can both shorten and lengthen within individuals. Longitudinal studies can potentially demonstrate associations between survival and telomere length, important in understanding population and individual-level effects. We studied individual house sparrows, *Passer domesticus*, in a wild, closed population, taking many repeated measurements of their telomeres (*N* = 1,494 measurements) across a 21-year period. On a longitudinal level, individuals experienced telomere attrition as they aged. Telomere length was not, however, associated with age across individuals. Such a difference in within-individual and between-individual age effects indicates that there has been selective disappearance, where individuals with shorter telomeres have disappeared from the population. Individuals did not show high consistency in their telomere lengths, and we provide evidence that telomere measurements increased in some individuals. If shortening and lengthening of telomeres can occur in response to environmental circumstances, telomere length might be more plastic than previously thought, with changes within individuals reflecting past and current life experiences.

3.2 Introduction

Senescence, defined as a reduction of residual reproductive success at older ages along with an increased likelihood of death, occurs in most organisms (Williams 1957; Kirkwood & Rose 1991). The main selective pressure that allows senescence to occur is extrinsic mortality, which selects for investment in early reproduction. This selection pressure results in a reduction in the strength of selection to maintain phenotypic traits as an individual ages, and results in a trade-off between investment in early-life reproduction and survival later in life (Williams 1957; Reznick *et al*. 2004). Evidence for this trade-off between early-life reproduction and growth or somatic maintenance has been found in a wide range of species (Lemaître *et al*. 2015; but see Cohen *et al.* 2020).

However, the exact costs that underlie trade-offs that mediate senescence are still largely unknown (Flatt & Hayland 2011; Young 2018). Only when the molecular and physiological constraints that

drive senescence are uncovered can we start to shed light onto this major knowledge gap (Vleck & Haussmann 2007). There has, therefore, been much recent interest in biomarkers or mechanisms of ageing that can be measured in an ecological context, and telomere biology specifically (Spurgin *et al*. 2018). Telomere length has received attention because telomeres act on a cellular level and their lengths, and changes in the lengths, have been linked with the health and survival of individuals (Wilbourn *et al*. 2018). As such, telomeres may offer a potential link between cellular processes and ageing (Monaghan & Haussmann 2006).

Telomeres are repeated sequences $(TTAGGG)_n$, located at the ends of chromosomes (Blackburn 2000). Telomeres have several functions within a cell. First, telomeres safeguard chromosome integrity by protecting chromosomes from nucleotide loss during cell division, as the ends of chromosomes are not fully replicated during DNA replication (the end replication problem, Blackburn 1991). Second, telomeres have been suggested to protect DNA from cellular oxidative stress, which shortens telomeres and would otherwise damage genes within chromosomes (Bize 2009; Blackburn *et al.* 2015). Third, short telomeres may trigger cellular responses when they reach a critically short length, potentially leading to apoptosis (Harley *et al*. 1990; Lansdorp 2000). More recently, it has been shown that telomeres are a hub for cellular signalling to detect damage to the cell, irrespective of their length, which can feed into apoptosis and other key cellular signalling pathways (Hemann *et al*. 2001; Simons 2015 and Hewitt *et al*. 2012; Victorelli & Passos 2017). Telomeres have been observed to shorten with age in many species (Dantzer & Fletcher 2015) and have been linked to lifespan and survival (overview in Wilbourn *et al.* 2018, and see in contrast Li *et al*. 2020). Due to this link between telomere length and lifespan and survival, it has been proposed that telomeres are biomarkers of age (Bekaert *et al.* 2005; von Zglinicki & Martin-Ruiz 2005; but see Mather *et al*. 2011), or more specifically of 'biological age' (Monaghan & Haussmann 2006, but see Boonekamp et al. 2013).

Oxidative stress has been hypothesised to account for the link between telomere length and aging (Reichert & Stier 2017). Oxidative stress arises when reactive oxygen species (ROS) are generated during, among other causes, organismal stress (Chatelain *et al*. 2020). ROS are highly reactive (Balaban *et al*. 2005) and can cause damage to telomeres, so shortening them (von Zglinicki 2002). As telomeres may be damaged by oxidative stress, and oxidative stress can arise from organismal stress, telomeres may be a potential measure of the ecological stress (and thus accumulated damage) that an individual has undergone in its lifetime (Monaghan & Haussmann 2006; Asghar *et al*. 2015; Bize *et al.* 2009), reflecting biological age (the age of an individual shaped by its lifetime

experiences), rather than chronological age. In line with the predictions following from this hypothesis is the observation that there is a large amount of variation in telomere length among individuals of the same species and age (Monaghan & Haussmann 2006; Hares *et al*. 2018). Such variation in telomere length between individuals of the same age suggests that individuals may undergo telomere shortening processes - such as stressful periods - differently (McEwen & Wingfield 2003; Pauliny *et al.* 2006) and, or alternatively, start life with different telomere lengths (but note that heritability estimates range from 0 to 1, Dugdale & Richardson 2018).

Evidence for variation in within-individual telomere attrition rates is scarce because within-individual differences can only be detected in longitudinal studies. Longitudinal studies are rare due to the time-consuming task of collecting multiple samples from individuals across their lifespans, and ideally need to be undertaken in a closed population, or dispersal may bias conclusions (Barrett *et al*. 2013). Cross-sectional studies cannot distinguish changes in a trait with age within individuals from a change in composition of a population. It is vital to distinguish between such longitudinal and cross-sectional effects because population-level associations between a trait and age can be caused by a completely different mechanism to within-individual associations (Reid *et al*. 2010; van de Pol & Verhulst 2006). A population's composition can change due to the selective disappearance over time of individuals that express a certain trait (Nussey *et al*. 2011, Lindström 1999). When low-quality or inferior individuals disappear from the population, the older cohorts will consist of a 'biased' population that expresses the trait that enabled them to survive (Nussey *et al*. 2011). If selective disappearance is not taken into account, within-individual changes in a trait will be underestimated (van de Pol and Verhulst 2006).

The importance of longitudinal studies is highlighted by mounting evidence that telomere length can both decrease and increase within individuals (Bateson & Nettle, 2017; Spurgin *et al*. 2017; Fairlie *et al*. 2016; Foley *et al*. 2020). Elongation of telomeres was an unexpected finding, given that telomeres were hypothesized only to shorten. Telomeres can be lengthened by an enzyme called telomerase (Blackburn *et al*. 1989). Contrary to elongation *per se*, elongation can also appear to be the case due to a change in blood cell turnover because telomere length is measured as an average across cells in a sample. Which of the possible elongation mechanisms operates is still unclear, but telomere elongation, especially when influenced by environmental conditions, may suggest that telomere length may not reflect accumulated costs accurately (Bateson 2016).

But why then, if telomeres can elongate, has selection not favoured longer telomeres in all species? Young (2018) provided two non-mutually exclusive hypotheses: the costly maintenance hypothesis

and the functional attrition hypothesis. The costly maintenance hypothesis incorporates the notion that telomeres are part of a trade-off, as the maintenance of telomeres may entail costs. However, considering that telomeres are a small fraction of an entire genome, the costs of maintaining telomere length may be relatively small. Moreover, many species have large sections of so-called 'junk DNA', which is called as such due to its apparent lack of protein coding DNA (Comings 1972), and the lack of a functional role at organism level (although non-coding DNA can potentially still serve a purpose; Palazzo & Gregory 2014). If junk DNA serves little or no purpose, it can be questioned why it persists in the genome, which consequently may imply that eliminating 'junk DNA' is costly, or the costs of maintenance of DNA can be low (Young 2018). In the case of the latter, it can be speculated that the maintenance costs of telomeric DNA can be negligible. However, it should be noted that even small maintenance costs may cause evolution to 'favour' telomere attrition, if the role of telomeres in life-history trade-offs is also small (Young 2018).

The functional attrition hypothesis proposes that telomere loss could be adaptive if the attrition itself has a purpose, such as preventing cancer (Young 2018). Telomeres prevent chromosome ends being repaired by DNA repair mechanisms (Campisi 2013). The prevention of DNA repair is vital in the telomeres, because if the repair of DNA is followed by a cell division, the genome will become unstable (Rodier *et al.* 2005). Genomic instability is a cancer risk. As such, when cells divide without telomerase (as may occur in cancerous cells), telomeres may become unstable, which triggers a cellular response that may lead to cellular senescence (Fumagalli *et al*. 2012; Campisi 2013).

If telomeres are part of the trade-off network, there may be evolutionary implications when the lengths of telomeres affect life-history trajectories and individuals suffer fitness consequences. Some life-history components have already been linked to telomere length. For example, telomere length at a young age predicts lifespan and survival in several species, including humans (Joeng *et al*. 2004; Haussmann *et al*. 2005; Salomons *et al*. 2009; Boonekamp *et al*. 2013) and birds (Haussman *et al.* 2005; Heidinger *et al.* 2012; Eastwood *et al*. 2019). It should be noted that the previous studies have shown associations between telomere length and survival. Whether telomere length is affecting survival directly, or is a biomarker of other processes that affect survival, is unclear (Simons 2015), but both possibilities are equally interesting. However, before attempting to study the exact significance – causal or as a biomarker – of telomeres in the aging process, we first need to understand how age and survival are potentially connected to telomere length, as this is a vital first step to shedding light on the possibility of telomeres affecting (or being a biomarker of) life-history components.

We studied the association between telomere length and age or survival using a large longitudinal study in a natural closed population of a bird, the house sparrow, *Passer domesticus*. We were able to separate associations of telomere length within and between individuals with age using longitudinal data collected over 15 years. We also have detailed age and survival information in this insular population. Telomere dynamics have previously only been studied in island populations of two birds and one mammal: Seychelles warblers, *Acrocephalus sechellensis*, house sparrows and Soay sheep, *Ovis aries* (Spurgin *et al*. 2017, Ringsby *et al*. 2015; Fairlie *et al.* 2016).

We focussed on the following hypotheses, based on the current literature: 1. Telomere length is negatively associated with age. 2. Some individuals show telomere elongation, and we therefore also expect low repeatability of telomere length within individuals. 3. Survival is positively associated with telomere length. 4. Telomere length is positively associated with lifespan.

3.3 Methods

3.3.1 Study system

We studied house sparrows on Lundy Island (51.11 N, 4.40 W), in the south-west of England, in 2001–2017. House sparrows are small, multi-brooded passerines (Summer-Smith 1963). Due to the remote location of the island and the sedentary nature of house sparrows (Bengtson *et al*. 2004), immigration and emigration are rare and estimated at 0.5% of all recruits (Schroeder *et al*. 2015). The birds breed in nest-boxes located mainly in the Lundy village (Griffith *et al.* 1999, Sánchez-Tójar *et al.* 2017).

Due to continuous catching efforts, almost all individual sparrows are uniquely colour ringed and provided with a metal numbered ring from the British Trust for Ornithology. Adults are caught in both summer (April – September) and winter (November – March) using mist nets, trapped within nest-boxes or with funnel traps. In our population, individuals are captured repeatedly throughout their lives. The age of all birds (in years) is known as almost all (>99%) of the house sparrows are banded as nestlings or first-year juveniles (Schroeder *et al.* 2015).

3.3.2 Telomere length

Blood samples used for telomere analysis were collected from juveniles (*i.e*. offspring that had fledged from the nest) and adults by puncturing the brachial venipuncture with a 26G needle and a

nonheparinized capillary tube. The blood from each individual was stored in 1 ml of *ca* 100% ethanol at room temperature. DNA was extracted using an ammonium acetate precipitation method (Nicholls *et al*. 2000; Richardson *et al.* 2001) and stored at -20◦C in low TE pH7.5.

DNA was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Fisher) and normalised to 20–30 ng/ul. DNA was extracted using an ammonium acetate precipitation method (Nicholls *et a*l. 2000; Richardson *et al*. 2001) and stored at -20◦C in low TE pH 7.5. For qPCR, a multiplex protocol was implemented following Cawthon (2009). Telomere length was measured in a StepOnePlus (Applied Biosystems) or Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). For specific details regarding the telomere measurement protocol, see Section 2.3.3.

The collection of blood samples over a range of years may lead to samples being clustered during analysis by, for example, year or cohort (van Lieshout *et al.* 2020). This may lead to different conditions (i.e. laboratory conditions) among clusters (van Lieshout *et al*. 2020). To avoid blood samples being biased by such clustering, all plates used for qPCR (quantitative polymerase chain reaction) contained a random subset of samples from three different years. For a detailed description of how the analysis was completed in batches, see Section 2.3.3.

We analysed 1,339 telomere length measurements from post-fledging (individuals that left the nest) house sparrows. These samples were from 853 individual birds from 21 cohorts (year of hatching). 557 individuals had one RTL measurement and 782 individuals had two or more measurements obtained following independent capture events. The total number of individuals or samples varies between analyses due to missing or uncertain values of age or lifespan. Uncertainty or missing age and lifespan values occur when an individual was not caught in its first year of life and the age had to be estimated. In this dataset, the average house sparrow age at sampling was 0.97 (SD 1.27) years. The oldest bird sampled in our population was 7 years old. The average lifespan of the birds sampled was 2 years (SD 1.7).

3.3.3 Storage time effects

As for any laboratory analysis when samples have been stored, there is a risk of degradation or loss of sample integrity. For telomeres specifically this issue has attracted attention, as change over time is often the focus of study. Blood sample storage time has been suggested to affect telomeres when stored at above freezing, where telomere measurements increased over time (Rosero-Bixby *et al*. 2019). In order to assess if storage time affected our telomere measurements, we developed an

experiment where we re-extracted the DNA and re-measured (log) relative telomere length in a randomly selected subset of 80 samples from all blood samples that we collected between 2000 and 2015. We detected a negative quadratic effect of the time a blood sample was stored in ethanol at room temperature (see Section 2.4). In the first five years of storage, blood sample storage time showed a negative linear association with telomere length, after which the association between storage time and telomere length became less strong. As the first 5 years showed a negative linear effect, we decided to only include blood samples that were stored for five years or less in statistical analyses. Moreover, we accounted for the blood sample storage time effect by including blood sample age (in years, as an integer) as a fixed covariate in all models.

3.3.4 Age of birds, lifespan, and survival

The ages of individual birds were calculated in years, based on their cohort (year of birth). On Lundy, the chance of resighting an individual is high (96%, Simons *et al*. 2015a). Resighting is done by using actual catches, sightings during the breeding season and winter, sightings from social parentage using video recording, and genetic sightings (Simons *et al*. 2019). The lifespans of birds were calculated by subtracting cohort from the year in which an individual was last observed alive. Due to the high resighting rate, we considered birds to be dead when not sighted for 2 years. Birds that were still alive at the time of analysis were excluded from analysis for the calculation of lifespan. Survival (binary) was defined as an individual living for at least another year after a telomere measurement was taken.

3.3.5 Statistical analysis

All analyses were conducted in the R environment (version 3.5.0; R Core Team 2018) and the 'lme4' package (version 1.1–21) was used to run the statistical models. A linear mixed model was used for all analyses, unless specified otherwise. After running an LMM, assumptions about homoscedasticity, collinearity and residual normal distributions were checked by visually inspecting residual plots. Relative telomere length was log transformed for all models, so the residuals fit a normal distribution, and will be referred to RTL hereafter. We considered an effect to be statistically significant when $p < 0.05$. P-values were calculated using the package LmerTest (version 3.1-0), using Satterthwaite's degrees of freedom.

3.3.6 Age effects

We tested whether RTL was associated with age. We separated cross-sectional (between-individual) and longitudinal (within-individual) effects using van de Pol & Verhulst (2006) methods, using within-

subject centring of age. To test the association of age (integer, in years) with RTL, we used RTL as a response variable, with the mean individual age (between-individual effect) and centred individual age (within-individual effect) as predictor variables. To test for selective disappearance in our population, we tested if the within-individual and between-individual slopes were significantly different. To test this, we applied van de Pol & Wright's (2009) method (equation 3). In this model, the centred age is replaced with our original fixed effect (age), while all other variables remained the same. Here, age, reflecting both within- and between-individual effects, now tests for within-individual effects because the model tests each fixed effect, while controlling for other effects in the model, including mean age. The mean age now represents the difference between the mean age and centred age slopes. If the estimate of mean age in this model is close to zero and nonsignificant, the within- and between-age effects on RTL are regarded to be similar.

Adding the year of hatching (cohort) to the models would provide information about potential differences in telomere length that occur due to the year of birth (Fairlie *et al.* 2016). However, we were unable to separate cohort from blood sample storage time effects, because the age of the blood sample was also included in the model, and cohort and sample age were correlated. We therefore excluded cohort from our analyses, with the consequence that we were unable to assess whether telomere length was affected by cohort.

Because associations between telomere length and age might be dependent on the sex of the bird, sex was added as a fixed (factor) effect, with an interaction with both centred age and mean age. Birds were repeatedly measured, and multiple samples were analysed on the same qPCR -plate; we accounted for this interdependence in the dataset by including the individual bird identity and qPCR plate identity as random intercepts.

To test if the effect of age on telomere length was different for individual birds, a random slope was assigned to centred age and bird identity. For this model, multiple measurements per individual were needed, and thus individuals that had two or more measurements were selected. The model that included the random slope for bird identity and centred age was compared to a model that did not include the random slope, using the Akaike Information Criterion (AIC). In order to make the model comparison, both models needed to use the same dataset, so the model that did not contain the random slope was also restricted to individuals with two or more measurements.

Additionally, we tested whether telomere lengthening occurred in some individuals in the population using Simons *et al.*'s (2014) statistical approach that separates true lengthening from the statistical noise expected from estimated error. This method fits regressions of age against RTL for each individual separately, and thus can only be used on individuals for which there are three or more measurements. We used residual RTL, as used in the Cox models (see Section 3.3.7. below), to fit individual regressions. Simons *et al.*'s (2014) approach uses several steps to calculate and compare the error variance (the variance due to measurement error) under different assumptions. The first assumption is that the RTL slopes describing change within individuals vary, and that telomeres thus increase and/or decrease. The error variance under this assumption is calculated (equation 4 in Simons *et al.* 2014). The second assumption is that telomeres do not elongate; the error variance under this assumption is also calculated (equation 5 in Simons *et al*. 2014). When the estimated error variance under the assumption that telomeres do not elongate (equation 5 in Simons *et al*. 2014) is higher than the error variance under the assumption that telomeres do increase and/or decrease (equation 4 in Simons *et al*. 2014), telomeres show elongation (Simons et al. 2014). A variance ratio test between the two variances was used to test whether the variances were significantly different (equation 6 in Simons *et al*. 2014). We used Equation 7 in Simons *et al*. (2014) to identify which individuals showed an increase in telomere length with a 95% confidence interval.

3.3.7 Survival and lifespan effects

We tested whether RTL was associated with post-fledging survival, using a Cox time-dependent regression. This model compares covariate values of individuals undergoing the event, in this case death, with values of other individuals who were present at that time. The time-dependent model uses time steps, which were the numbers of days between successive captures, with the first time set as the number of days between hatch date and first capture. The exact hatch dates for some individuals were unknown, so we calculated the median hatch date (from all individuals in our dataset) and set that date (13 June) as the date of hatching for all individuals.

For analysis, we knew that RTL was affected by the blood sample storage time and the qPCR plate. Thus, using RTL as a predictor for survival in a Cox model posed the problem that these factors could not be accounted for. Therefore, to use RTL as a predictor, we first used a linear mixed model with RTL as the response and corrected for blood sample age (added as a fixed effect) and qPCR plate (added a random intercept) effects. The residuals from the linear mixed model were then used as the predictor for survival in a Cox time-dependent regression model. Our most recent samples were

collected in 2015, meaning that for all birds the survival (living for at least a year or more after capture) was known, because monitoring the population continued until the time of analysis (2020).

To test if lifespan was associated with early-life telomere length, we used the first RTL measurements of young birds (younger than 1 year). A Cox proportional-hazard model was used, where the RTL residuals from the linear mixed model (as used for the Cox time-dependent regression model, see above) was used as the predictor variable. The lifespan (as an integer, in years) of individuals was added as the response variable. Twenty-nine birds were still alive at the time of analysis (2020). These birds were right censored.

3.3.8 Repeatability of individuals

We analysed individual bird repeatability in telomere length using the rptR package (Stoffel *et al.* 2017). rptR uses a mixed linear model framework, where the repeatability is calculated as the variance among the means of groups (in our case individual birds) over the sum of group-level and data-level (residual) variance. In rptR we used RTL as the dependent variable and individual age (integer, in years) and blood sample storage time (integer, in years) as covariates to account for age and storage time effects on telomere length. qPCR-plate was added as a random effect.

3.4 Results

3.4.1 Mean and centred age effects

The sexes of birds were not related to RTL ($\beta_{sex\ (male)}$ = -0.01; se = 0.02, t = 0.35, df = 655; p = 0.73) and neither the association between centred age nor mean age on RTL was dependent on sex ($\beta_{sex*centred\ age}$ = 0.02, se = 0.03, t = 0.81, df = 566.6, p = 0.41 and $\beta_{sex*mean\ age}$ = 0.03, se = 0.02, t = 1.42, df = 562.9, p = 0.16, Table S3.1). Because the interactions between sex and centred and mean age were not significant, both interactions were removed from the model. We then tested whether the RTL of individuals was correlated with age and found no between-individual age effect ($\beta_{mean\ age}$ = -0.00, se = 0.01, t = -0.13, df = 543.5, p = 0.90). There was a significant negative within-individual effect of age on RTL ($\beta_{centred\ age}$ = -0.04, se = 0.01, t = -2.78, df = 706.1, p = 0.00, Figure 3.1 and Table 3.1).

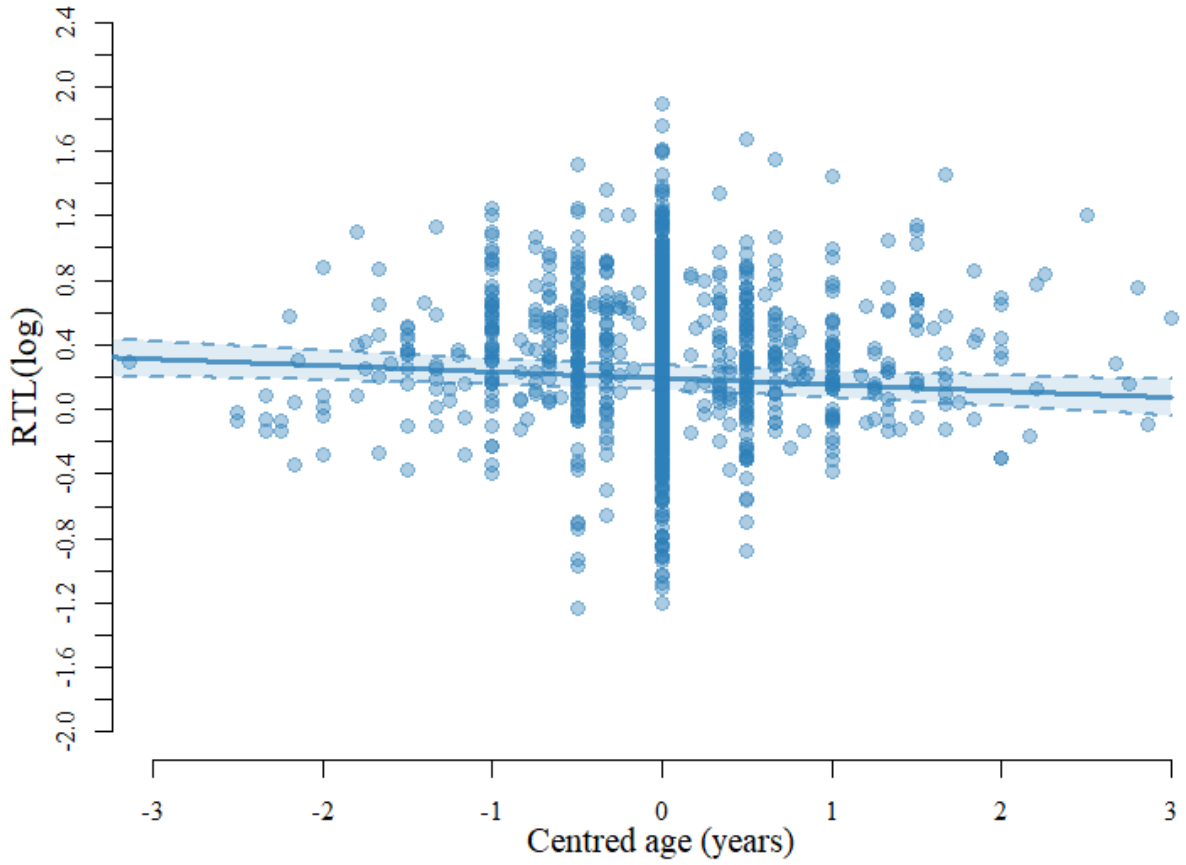


Figure 3.1. Within-individual age effects on telomeres in the house sparrow on Lundy. As individuals age, their telomeres mostly shorten. The solid line represents the model estimate, while the dashed lines represent the 95% confidence intervals. The dots are the raw data points.

Table 3.1. Mean and centred effects on RTL, from the linear mixed model.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.206	0.043	4.756	105.0	**<0.001**
Mean age	-0.001	0.010	-0.125	543.5	0.900
Centred age	-0.040	0.014	-2.782	706.1	**0.000**
Blood sample age	-0.019	0.012	-0.978	1286.0	0.328
Sex (male)	-0.002	0.020	-0.075	665.2	0.940
Random effects	**Variance**	**Stan. dev.**			
Bird identity	0.018	0.133			
qPCR plate identity	0.105	0.325			
Residual	0.095	0.308			

We tested if the between-individual (mean) age slope was significantly different from within-individual (centred) age slope, and found that the mean age and centred age slopes were significantly different ($\beta_{\text{Slope difference}}$ = 0.04, se = 0.02, t = 2.16, df = 1,252.0, *p* = 0.003, Appendix S3.2, Table S3.2).

3.4.2 Telomere length effects on survival and lifespan

There was a positive, non-significant, association between survival and residual RTL (log hazard = -0.06, se = 0.11, Z = -0.56, p = 0.58, N = 1304, N events = 748). Therefore, for an increase of 1 residual RTL, the chance of death was lowered by 6% (linear hazard = 0.94).

Young individuals (less than one year old) with longer RTL did have longer lifespans, but this effect was not significant (log hazard = -0.06, se = 0.14, Z = -0.42, p = 0.67, N = 541, N events = 514).

3.4.3 Variation within individuals

Individuals showed low repeatability, with only 11% of the variance in telomere length being explained by among-individual differences in RTL in a model that accounted for individual age, qPCR plate and blood sample age effects (R = 0.11, se = 0.03).

Including a random slope for centred age and bird identity, to test whether individuals had varying slopes, provided no better fit than a model with only a random intercept for centred age (AIC of 615.2 for the model without the bird identity and centred age slope, versus 616.4 for the model with only a random intercept for centred age). The individual slope model and the model that it was compared to are presented in Supporting Information (Tables S3.3 and S3.4). The model that allowed individuals to have varying slopes did not provide a better fit than a model that had fixed slopes for individuals, showing that there was no support for individual differences in the association between RTL and age.

We tested whether telomere lengthening could be attributed to measurement error, using Simons *et al*.'s (2014) approach. This test showed that increases or decreases could not be attributed to measurement error (estimated error variance = 0.02, p < 0.01, F variance test). However, only 7 individuals showed significant elongation (out of 115 individuals). To visually illustrate that telomeres can elongate in some individuals, we selected all individuals with 5 or more telomere measurements (Figure 3.2).

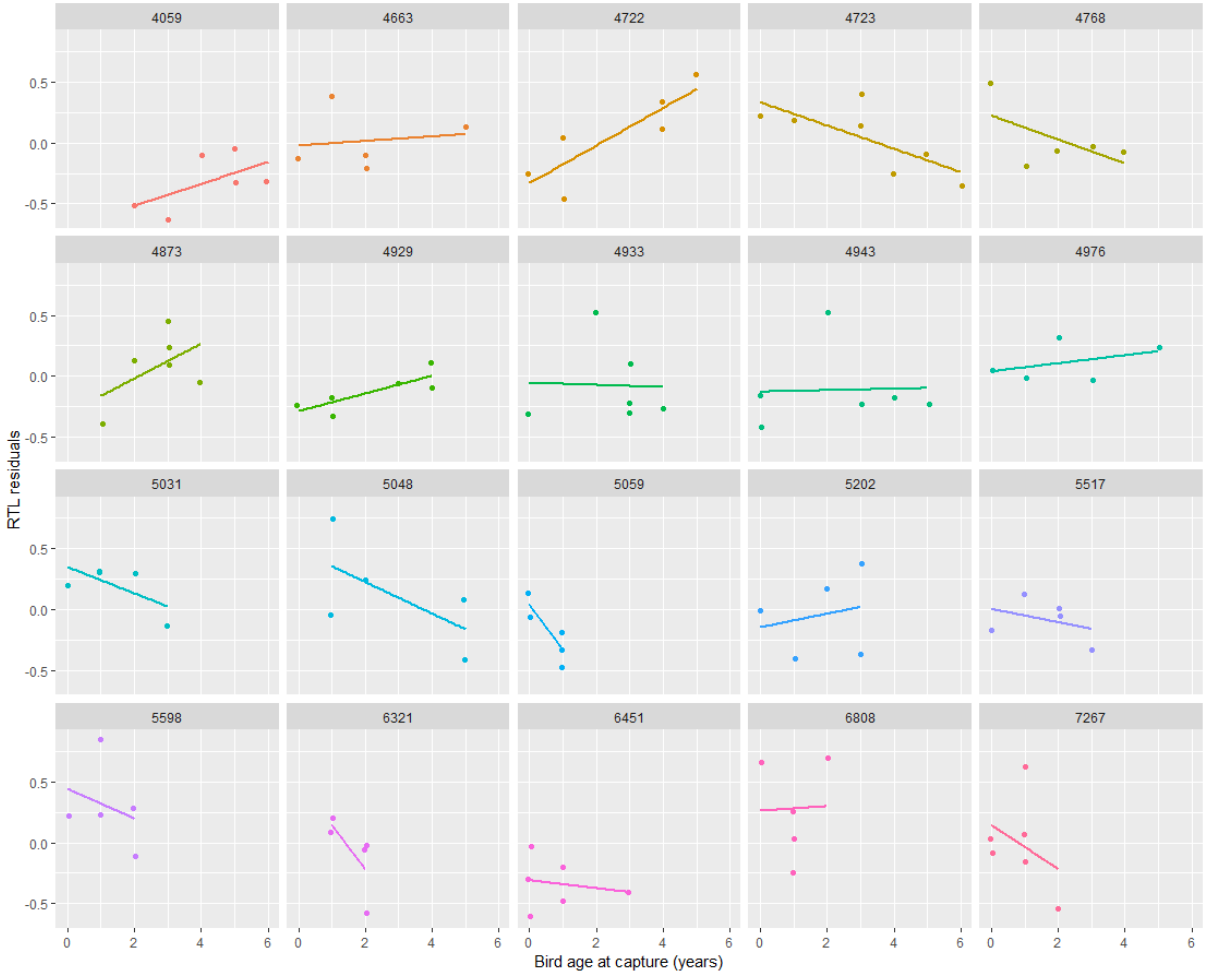


Figure 3.2. Linear regressions of 20 individuals (their individual identities are shown at the top of the plots) that each had five or more telomere measurements. The x-axis shows the age of the bird at capture (in years) and the y-axis shows residual RTL from a model correcting for qPCR plate and blood sample age effects.

3.5 Discussion

We used one of the largest longitudinal datasets to date, with only the Seychelles warblers study obtaining more telomere measurements (1,808 telomere measurements from 1,040 individuals, Spurgin *et al*. 2018), to assess the relationship between lifelong telomere dynamics and age, adult survival and whether early-life RTL is associated with longevity within a wild closed population. We found a positive but non-significant association of telomere length with age, which is contrary to previous studies, which mainly detected a decline of telomere length with age at the population level (Pauliny *et a*l. 2006; Salomons *et al*; 2009; Spurgin *et al*. 2018). Population-level effects are interesting, as they signify that telomeres may be used as a biomarker of age (either calendar or biological). Telomeres that reflect age adequately could help in providing age estimates of individuals in the wild, as obtaining known ages can be difficult in wild populations (Nakagawa *et al*.

2004). However, not all studies report population-level effects (Hall *et al*. 2004), and sometimes associations of telomere length with age were complex and driven by, for example, variation among cohorts (Young *et al.* 2013). We were unable to separate cohort effects from blood sample storage time effects, so we do not know how cohort might have affected associations of telomere length with age in our house sparrow population.

3.5.1 Mortality and selective disappearance

We find that telomere length generally shortens with age within individuals. This is in line with other studies that also concluded that telomeres shorten within individuals with increasing age (Hall et al. 2004; Pauliny *et al*. 2006; Salomons *et al*. 2009; Young *et al*. 2013; Fairlie *et al*. 2016, Spurgin *et al*. 2018). The lack of an association between age and telomere length between individuals, in comparison to a negative association within individuals, indicates selective disappearance (Snijders & Bosker 1999). In our case, selective disappearance acts on individuals that have short telomeres, making them disappear out of the population with age, resulting in a population with relatively longer telomeres at older ages (Fairlie *et al*. 2016; Spurgin *et al*. 2018). However, it should be noted that we have not provided evidence for a causal relationship between telomere length and the disappearance of individuals. It is plausible that telomere length reflects other processes that may lead to the disappearance of individuals, instead of causing disappearance.

Regardless, whether telomeres cause selective disappearance or are a biomarker of intrinsic processes that lead to selective disappearance, these results imply that birds with shorter telomeres should have lower survival. Not finding survival effects in our Cox model is in contradiction to what was previously found in a range of studies and species (Wilbourn *et al*. 2018). We propose two possible explanations: 1. Attrition of telomeres affects survival instead of telomere length. 2. A difference in timing of when the first telomere measurement was taken.

The first explanation of why we did not detect survival effects is due to our study using telomere length instead of telomere attrition as the predictor of survival. Attrition may affect survival through two mechanisms: 1. Telomere attrition may lead to the accumulation of senescent cells and the depletion of stem cell stocks, which accelerates tissue damage and in turn might affect survival (Boonekamp *et al*. 2013; Herbig *et al.* 2006; Campisi & Fagagna 2007). 2. Higher telomere attrition might reflect accumulated damage in the body, which in turn affects fitness and survival (Metcalfe & Alonso-Alvarez 2010; Young, 2018). Two animal studies to date investigated the relationship between telomere attrition and length, and consequent survival to recruitment age, and both

concluded that only attrition affected survival negatively (Boonekamp *et al*. 2014; Wood & Young. 2019). In humans, a meta-analysis showed that there was an association between mortality and telomere length, but the study also concluded that telomere attrition may more closely predict mortality, especially at older ages (Boonekamp *et al.* 2013).

Second, is a difference between some of the studies that researched telomere effects on survival and our study is the timing of the telomere measurements. We took the first telomere measurements after fledging. In Heidinger *et al*. (2012)'s and Eastwood *et al*.'s (2019) studies, birds were measured as nestlings. Telomere dynamics may be vastly different in nestlings than in adults. For example, attrition rates tend to be higher early in life (Hall *et al*. 2004; Fairlie *et al*. 2016). Such declines are usually most marked during periods of an individual's life when growth is occurring (Pauliny *et al*. 2015). During growth there is high cell proliferation, which can cause telomere shortening (Zeichner *et al.* 1999). Moreover, nestlings can experience stresses, such as food shortage, that impact growth, and consequently telomere dynamics. The impact of stress on telomere dynamics was demonstrated in jackdaw, *Corvus monedula,* nestlings (Boonekamp *et al*. 2014). These nestlings were exposed to increased or reduced stress by increasing or decreasing brood sizes, which resulted in longer telomeres in nestlings in reduced broods and shorter telomeres in increased broods. The effect on telomere lengths was still apparent later in life and the attrition rates predicted post-fledging survival. Due to taking measurements from post-fledglings, we may not have picked up on any effect, potentially strong, on survival that may have occurred in the nestling period.

We found no significant association between telomere length and adult survival in our Cox model, which seems contrary to finding selective disappearance. As our population of house sparrows is insular, any effects of telomere length on survival should be detected, as birds cannot leave the island and thus our data are not affected by individuals leaving the study site. A potential explanation why we did not detect survival effects in adults in our Cox model, but do find selective disappearance when comparing within-individual and between individual slopes, is catching bias, which might cause a shift in the between-individual and within-individual slopes if, for example, older individuals are more likely to be caught. The slope of the between-individual effect will then be driven upwards at older ages, increasing the difference between the within-individual and between-individual slopes. Selective disappearance would then be wrongly concluded as occurring, when it did not. However, there is limited catching bias in our house sparrow population (Simons *et al*. 2015a), so it is unlikely that a catching bias is the cause of detecting selective disappearance.

It should be noted that the results of the Cox models are nonetheless in the direction of expectation (*i.e*. less survival and shorter lifespans in individuals with shorter telomeres). Moreover, a recent meta-analysis from Wilbourn *et al.* (2018) assessed 27 survival studies and concluded that there was an association between survival and telomere length. The mean log hazard ratio from these 27 survival studies was 0.21 (s.e = 0.05), which is similar to our log hazard of 0.16. It thus remains likely that survival is associated with telomere length in adult house sparrows.

3.5.2 Telomere lengthening

Some individuals showed possible elongation of telomere lengths that could not be explained by measurement error. In contrast, our random slope model, that allowed the slopes of individual birds to vary with age and RTL, did not provide a better fit than a model that did not include random slopes for individual birds. This contradiction can potentially be explained by the few birds that showed significant elongation in our study (9 out 141 individuals). The majority of birds, however, show within-individual telomere attrition with age, which explains why the random slope model did not provide a better fit.

Telomeres can elongate due to a ribonucleoprotein enzyme called telomerase, and to a lesser extent recombination (Kass-Eisler & Greider 2000). Telomerase has been found to elongate telomeres in several species, including humans. In humans, telomerase is expressed in embryos, but down-regulated in most tissues in adults (Meeker & Coffee 1997; Haussmann *et al*. 2004). However, in contrast, telomerase is expressed in both embryonic and adult rodents, such as laboratory mice, or long-lived birds, such as the Leach's storm petrel (*Oceanodroma leucorhoa*; Kipling & Crooke 1990; Haussmann *et al*. 2004). It is thus possible that telomerase is also expressed in (some tissues of) house sparrows.

There are benefits associated with telomere elongation. It is not the average telomere length, but the shortest telomeres in a cell population, and indeed among chromosomes within a cell, that are likely to have the most impact on cellular survival (Hemann *et al*. 2001). Senescence has been hypothesized to be induced by critically short telomeres, and thus elongation of telomeres would reduce the number of cells with critically short telomeres and thus senescence (van Deursen 2014). In addition, as telomere elongation can be the result of telomerase activity, among other mechanisms, telomerase itself has been associated with regeneration of tissue and, in mice,

beneficial to health parameters (Reichert *et al.* 2014; Simons 2015). However, elongation can also come at a cost, as elongation itself may be energetically costly, or pose a cancer risk (Young 2018).

The potential cancer risk associated with telomere maintenance or elongation may be an insignificant risk in our house sparrow population. House sparrows are relatively short-lived and experience high extrinsic mortality factors (as indicated by the recruitment levels, which are around 20%; Cleasby *et al.* 2010). The potential risk of rescuing critically short telomeres through the use of telomerase – tumours – may be of an insignificant risk as birds may perish before tumours become an issue.

Aside from true elongation, telomere elongation may be attributed to measurement error (Steenstrup *et al*. 2013), or telomeres may only appear to elongate. However, Simons *et al*. (2014) provided us with a statistical test that we could use to show that the increase in telomere length in our data is not attributable to measurement error. However, it is still possible that telomeres did not elongate. Telomeres are measured as an average telomere length of a population of blood cells in a sample. Consequently, elongation of telomeres within individuals may not truly reflect a lengthening of telomeres, but instead reflect a change over in cells, where cells with shorter telomeres are expelled, leading to overall longer telomere measurements (Spurgin *et al*. 2018). Such a change in the population structure of (blood) cells may be more likely in mammals, where telomeres are often measured in leucocytes, where leucocyte type (types have different telomere lengths, Weng 2012) proportions have been found to change in response to infection, in turn affecting telomere length measurements (Weng 2012; Beirne *et al.* 2014). In birds, telomere length is measured in nucleated erythrocytes, which have a rapid cell turnover with a maximum lifespan of 35 days in chickens, 45 in pigeons and 42 in ducks (Rodnan *et al.* 1957). House sparrows may have similar or shorter erythrocyte lifespans, and we can thus speculate that house sparrows can have swift changes in blood cell composition and, consequently, telomere length.

In addition to showing that some individuals showed telomere elongation, individuals showed low repeatability in RTL. Such a low repeatability is in agreement with what was previously found in the Seychelles warbler, where repeatability of telomere length in individuals was estimated to be 7% (Spurgin *et al*. 2018). Seychelles warblers, a species living up to 15 years of age, showed cross-sectional and longitudinal shortening of telomeres, but with bouts of telomere lengthening in some individuals, which was shown not to occur due to measurement error (Spurgin *et al*, 2018). These bouts of lengthening occurred mainly right after the juvenile period, around 4 years of life, and again

later in life (Spurgin *et al*, 2018). Telomeres were also highly inconsistent in telomere length across years and lifespans in a wild Soay sheep population (Fairlie *et al*. 2016). In this population of Soay sheep, telomere lengths could also elongate or show attrition, but this was associated with year, and thus environmental circumstances.

3.5.3 Conclusion

In conclusion, telomeres shorten within individuals, and individuals with short telomeres disappear out of our population. Individuals do not show a high consistency in telomere length, and some individuals show telomere elongation. The low consistency in telomere length, and the instances of telomere elongation, indicate that telomeres may be more plastic than previously thought. If telomeres show plasticity in their length, they may not then be a reliable biomarker of accumulated damage over time (biological age), at least in some individuals. However, we did find an overall within-individual decline in telomere length with age, suggesting that most individuals do undergo telomere attrition, and thus telomere length may, overall, still act as a biomarker of age.

Supporting Information S3

Table S3.1. Age and sex model with sex and age interaction

Effects of age, blood sample age and sex on Relative Telomere Length (RTL), showing linear mixed model results.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.220	0.044	4.951	117.2	**<0.001**
Mean age	-0.017	0.015	-1.152	501.7	0.250
Centred age	-0.051	0.020	-2.569	633.8	**0.010**
Blood sample age	-0.011	0.012	-0.939	1286.0	0.348
Sex (male)	-0.026	0.026	-0.974	812.5	0.330
Sex (male) * Centred age	0.021	0.026	0.810	566.6	0.418
Sex (male) * Mean age	0.027	0.019	1.423	562.9	0.155
Random effects	**Variance**	**Stan. dev.**			
Bird identity	0.018	0.133			
qPCR plate identity	0.105	0.324			
Residual	0.095	0.308			

Table S3.2. Mean age and centred age slope comparison

Effects of age, mean age and blood sample on Relative Telomere Length (RTL). The mean age here represents the difference between mean and centred age (van de Pol & Wright 2009).

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.206	0.043	4.756	105.0	**<0.001**
Age	-0.040	0.014	-2.782	706.1	**0.006**
Mean age	0.038	0.018	2.164	1252.0	**0.003**
Blood sample age	-0.012	0.012	-0.978	1286.0	0.328
Sex (male)	-0.002	0.020	-0.075	665.2	0.940
Random effects	**Variance**	**Stan. dev.**			
Bird identity	0.018	0.133			
qPCR plate identity	0.105	0.325			
Residual	0.095	0.308			

Table S3.3. Random slope for within-individual age comparison

Effects of age, mean age, centred age, blood sample age and sex on Relative Telomere Length (RTL). Only individuals with two or more measurements were selected. A random slope was added for centred age and individual.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.292	0.043	6.731	123.5	**<0.001**
Mean age	-0.027	0.013	-2.055	270.8	**0.040**
Centred age	-0.029	0.017	-1.734	114.5	0.086
Blood sample age	-0.010	0.015	-0.664	731.0	0.501
Sex (male)	0.043	0.029	1.519	255.5	0.130
Random effects	**Variance**	**Stan. dev.**			
Bird identity	0.020	0.147			
Bird identity | centred age	0.004	0.066			
qPCR plate identity	0.058	0.240			
Residual	0.087	0.295			

Table S3.4. Random slope for within-individual age comparison

Effects of age, mean age, centred age, blood sample age and sex on Relative Telomere Length (RTL). Only individuals with two or more measurements were selected. This model was used to compare to the random slope model, presented in Table S3.3.

Fixed effects	Estimate	se	t	df	*p*
Intercept	0.292	0.043	6.723	123.6	**<0.001**
Mean age	-0.028	0.013	-2.111	273.9	**0.036**
Centred age	-0.021	0.015	-1.388	629.2	0.166
Blood sample age	-0.008	0.015	-0.543	762.2	0.587
Sex (male)	0.042	0.029	1.490	256.2	0.138
Random effects	**Variance**	**Stan. dev.**			
Bird identity	0.020	0.140			
qPCR plate identity	0.058	0.242			
Residual	0.092	0.303			

4. Contrasting genetic and social paternal age effects on offspring telomere length

4.1 Summary

Parents shape the fate of their offspring not only through inherited genetics but also through social parenting effects. It has been increasingly recognised that parental effects can cause transgenerational effects on offspring fitness, in particular when parents age. Parental age effects on telomere length are a hypothesised mechanistic explanation of these transgenerational effects. Here, we separate the genetic and rearing effects of parental age on offspring telomere length, using cross-fostering in an insular population of house sparrows, *Passer domesticus*. This population of house sparrows has been studied for 20 years and individuals have been repeatedly sampled, which allowed us to separate within-individual from between-individual age effects.

Within genetic fathers, we found a negative effect of paternal age on offspring telomere length. We also found a population-wide effect, where older social fathers reared offspring with longer telomeres, while individual social fathers showed no increase in offspring telomere length with age. Moreover, we found no effect of moather age on offspring telomere length. We found evidence for selective disappearance for genetic and social fathers, respectively. We hypothesise that aging genetic fathers produced offspring with shorter telomeres because of an imperfect embryonic (telomeric) reset. Moreover, we propose that social fathers improved offspring telomere length with age due to a quality differences between fathers, where good quality fathers obtained better breeding sites early in life and may have been able to offset telomere attrition more effectively than low-quality fathers, consequently producing offspring with longer telomeres throughout their lifetimes.

4.2 Introduction

Senescence occurs when the accumulation of damage throughout life results in an accelerating decline of function across an individual's organs, leading to lower survival probability and offspring production (Jaskelioff *et a*l. 2011: Bouwhuis *et al*. 2012). Senescence is an aging effect, but the distinction between senescence and aging is that senescence is progressive and irreversible, leading to negative fitness effects, whereas aging may not necessarily lead to an impairment of fitness (Monaghan *et al*. 2008). Senescence is an evolutionary conundrum, because senescence is not beneficial to individuals and thus selection should act against it (Monaghan *et al*. 2008). Trying to

understand this evolutionary conundrum, the evolution of senescence and the underlying mechanisms, have been a topic of research for many decades (Gaillard & Lemaitre 2020).

In addition to senescence at the individual level, there is accumulating evidence of senescence having transgenerational effects. Older parents may influence the lifespans and reproductive success of offspring, called the Lansing effect (Lansing 1947; Bouwhuis *et al*. 2010; Ducatez *et al*. 2012; Schroeder *et al*. 2015). When transgenerational age effects are negative, an increase in selection pressure on longevity is expected, because older parents have reduced indirect fitness, which in turn can impact the age structure of a population (Schroeder *et al*. 2015). However, the mechanisms underlying such transgenerational effects remain unclear (Priest *et al*. 2002; Heidinger *et al*. 2016).

One mechanism that has been suggested to underlie the transgenerational effects of senescence is telomere length (Unryn *et al*. 2005; Heidinger *et al*. 2016; Eisenberg *et al*. 2019). Telomeres are repetitive DNA sequences located at the ends of chromosomes (Blackburn 1991). Telomeres protect chromosomes from losing coding sequence, maintain genomic stability and prevent the cell's repair mechanism from regarding the chromosome ends as DNA breaks (Blackburn 1991). Short telomeres have been correlated with reduced survival and disease incidence in animals, including humans (Haussmann *et al*. 2005; Aubert & Lansdorp 2008; Salomons *et al.* 2009; Butt *et al*. 2010; Wilbourn *et al.* 2018; Pepper *et al*. 2018). The lengths of telomeres are the outcome of lengthening and shortening processes. Telomeres can be elongated by the enzyme telomerase, but this enzyme is repressed in most somatic tissues in adult vertebrates (Greider & Blackburn 1985; Blackburn *et al*. 1989). There are other potential pathways that may result in telomere elongation (Mendez-Bermudez *et al*. 2012), however, an overall shortening of telomere length with age is observed in most species (Dantzer & Fletcher 2015). Telomeres shorten with each cell replication due to an imperfect DNA replication mechanism (Olovnikov 1973), and the attrition can potentially be accelerated by stress and adversity (Bize *et al*. 2009; Houben *et al*. 2008, Reichert *et al.* 2017; Pepper *et al.* 2018).

Variation in telomere length between and within individuals can be the result of differences in the aforementioned shortening and lengthening processes, but also due to a difference in initial telomere length. Telomere length can be heritable in some species (heritability estimates range from 0 to 1, overview in Dugdale & Richardson 2018). Parents may also potentially influence the telomere length of their developing offspring via the environment they provide, including the age of the parents (Ashghar *et al*. 2015). Offspring telomere length may be affected by maternal age-related

changes in the hormones or nutrients that they provide, which may also in turn be influenced by male age (Heidinger & Young 2020). In addition, the care a parent provides to its offspring may change with age, which in turn may affect offspring telomere length (Beamonte-Barrientos *et al*. 2010; Costanzo *et al*. 2016; Heidinger & Young 2020). The effects of parental age on telomere length can thus act through genetic parental effects or rearing parental effects, which can be experimentally separated using cross-fostering in wild birds (Kruuk & Hadfield 2007).

The age of males and females may affect offspring telomere length differently because females obtain all oocytes prenatally, while a male's sperm production is an ongoing process, which has been proposed to make sperm more prone to telomere attrition (Kimura *et al*. 2008; Keefe *et al*. 2015). However, even though oocytes are produced prenatally, this does not mean that telomere length in oocytes is unaffected by age. Oxidative stress can damage telomeres (Reichert & Stier 2017), and older gametes may have undergone more oxidative stress, resulting in shorter telomeres (Cimadomo *et al*. 2018). Moreover, it is speculated that eggs from older females underwent more mitotic cell cycles, which shorten telomeres, before entering meiosis during fetal oogenesis (Keefe & Liu 2007).

For males, due to the continuous replication of sperm cells, telomeres in sperm might shorten with male age. By contrasting, the continuous shortening of telomeres in sperm may also cause selection on sperm with longer telomeres, as sperm with short telomeres might disappear (Wallenfang *et al*. 2006; Froy *et al*. 2014; Eisenberg *et al.* 2018). Telomeres could even elongate in sperm, as germ cells are one of the few tissues that keep expressing telomerase (Kimura *et al*. 2008). The activity of telomerase is hypothesized to lead to a telomeric reset at conception, but to what extent telomere restoration occurs is still largely unknown (Vizlin-Hodzic *et al*. 2009). However, if a reset does not (fully) occur, male age may affect offspring telomere length at conception.

Effects of the rearing environment on offspring telomere length have been observed previously (Haussmann *et al*. 2012; Broer *et al*. 2012; Herborn *et al*. 2014; Costanzo *et al*. 2017), with an often observed pattern of accelerated telomere loss during periods of stress, such as reduced food or increased sibling competition (Boonekamp *et al*. 2014; Herborn *et al.* 2014). In many species, the age of a parent affects the quality of the breeding territory, or the ability to provision young (Froy *et al*. 2013; Nussey *et al.* 2013; Oro *et al.* 2014; Rabon 2014; Costanzo *et al*. 2017; Criscuolo *et al*. 2017). In turn, telomere dynamics in offspring have been correlated with the quality of parental care in some

species (Clutton-Brock, 1991; Haussman *et a*l. 2012; Broer *et al*. 2012). This means that parental age may affect offspring telomere length indirectly, via environmental circumstances.

However, linking parental age directly to telomere dynamics in offspring has led to mixed and contrasting results in a range of species, including birds, mammals and lizards (Heidinger & Young 2020). There appears to be an inconsistency when comparing birds, small mammals and lizards and humans and chimpanzees (Heidinger & Young 2020). In birds, two-thirds of the studies show a negative correlation between paternal and maternal age with offspring telomere length (Heidinger & Young 2020); this is in contrast to humans and chimpanzees, where there is a positive association between offspring telomere length and paternal age (Unryn *et al.* 2005; Kimura *et al*. 2008; Broer *et al*. 2013; Eisenberg 2018; Eisenberg 2019). The different parental effects on offspring telomere length among taxa may stem from both the study systems and study designs (Heidinger & Young 2020). One potential difference in study design is the use of cross-sectional versus longitudinal data, with many studies that have investigated the relationship between offspring telomere length and parental age having used the former (Bauch *et al*. 2019; Eisenberg *et al*. 2019). In cross-sectional studies, individuals are sampled once, which makes it impossible to assess if any relationship observed between offspring telomere length and parental age occurs within individuals, or is instead just a change observed at the population level (Van de Pol & Verhulst 2006).

We used a 15-year longitudinal dataset from a wild population of house sparrows, *Passer domesticus*, where a transgenerational negative fitness effect has been previously detected (Schroeder *et al*. 2015). Schroeder *et al.* (2015) found, at the cross-sectional level, that individual females with older mothers and individual males with older fathers produced fewer annual and lifetime recruits than individuals with younger parents. This negative effect of mother and father age was explained by the genetic parents – affecting offspring before one day of hatching, because offspring were cross-fostered at that age – while the age of the foster parents did not predict the fitness of offspring. Schroeder *et al*. (2015) proposed that telomeres could be a potential mechanism contributing to the negative genetic parental age effect on offspring fitness.

In the population of house sparrows on Lundy, offspring were routinely cross-fostered between nests at a young age, which allowed us to assess these questions: 1. Is there a relationship between telomere lengths of fledged offspring (offspring that left the nest) and parental age? 2. Is telomere length in fledged offspring best explained by the age of genetic parents (i.e. an effect arising due to genetics, incubation or very early life (one day) parental care) and/or by the age of the foster

parents (i.e. parental care and/or environmental effect after day one of hatching)? Due to the longitudinal nature of our data, we can distinguish within-individual from between-individual parental age effects on fledged offspring telomere lengths. This enables us to detect selective disappearance when the slope of the within-individual effect differs from that of the between-individual effect (van de Pol & Wright 2009).

4.3 Methods

4.3.1 Study system

We studied house sparrows on Lundy island (51.11 N, 4.40 W), in the south-west of Britain, studied in 2001–2015. House sparrows are small, multi-brooded passerines (Summer-Smith 1963). Lundy Island is located 20 kilometres from the coast, which given the sedentary nature of house sparrows (Bengtson *et al*. 2004), led to very low immigration and emigration (0.5% of all recruits, Schroeder *et al*. 2015). The birds breed in nest-boxes located on the island in sheds and workshops (Griffith *et al*. 1999, Sánchez-Tójar *et al*. 2017).

All individual sparrows were uniquely colour ringed and provided with a metal numbered ring from the British Trust for Ornithology (BTO). Adults are caught in both summer (April–September) and winter (November–March) using mist nets or trapping within nest-boxes and funnel traps. Individuals were captured and blood sampled repeatedly throughout their lives. The ages of all birds (in years) were known as almost the entire (>99%) house sparrow population were banded as nestlings or first-year juveniles (Schroeder *et al.* 2012).

In house sparrows, both males and females incubate the eggs and take care of the young. However, males incubate half as much as females (Bartlett et al. 2005) and provide 25% less food deliveries than females in broods that are older than one week (Mock *et al*. 2005). About 17.5% of all offspring in the Lundy house sparrow population are the product of an extra-pair mating, while 38% of all broods contained at least one extra-pair offspring (Hsu et al. 2015). Moreover, male sparrows on Lundy adjusted their offspring provisioning rates according to being cuckolded by females, but their incubation efforts remained unaffected (Schroeder *et al.* 2016).

4.3.2 Cross-fostering

Because we were interested in genetic parent effect (the parent that provided the egg or sperm) versus social (the parent that took care of the chicks) parental effects on offspring telomere length, we used a cross-fostering experiment where nestlings were cross-fostered reciprocally one day after hatching into any available brood of the same hatching date. If no brood was available of the same age, the brood was not cross-fostered. Broods were never reduced or enlarged in size. This meant that if two broods with an unequal number of chicks were available for cross-fostering, not all chicks were cross-fostered (Winney *et al*. 2015).

4.3.3 Parentage

We used data from 13 microsatellite markers amplified in two multiplexes (Dawson *et al.* 2012) to assemble a genetic pedigree of our population and so assign genetic parents (Schroeder *et al*. 2012). Social parents were identified for each brood via video recordings of the nest at day six of incubation, where the unique colour combination on the legs of each parent was noted.

4.3.4 Age of parents

For individual offspring, the age of the father and mother were calculated in years (integer) by deducting the parent's year of hatching (cohort) from the year in which the offspring hatched.

4.3.5 Telomere length

Blood samples were collected from juveniles (*i.e.* offspring that had fledged from the nest) and adults by puncturing the brachial venipuncture with a 26G needle and collection with a nonheparinised capillary tube. The blood from each individual was stored in 1 ml of *ca* 100% ethanol at room temperature. DNA was extracted using an ammonium acetate precipitation method (Nicholls *et al*. 2000; Richardson *et al.* 2001) and stored at -20°C in low TE pH 7.5.

DNA was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Fisher) and normalised to 20–30 ng/ul. DNA was extracted using an ammonium acetate precipitation method (Nicholls *et a*l. 2000; Richardson *et al*. 2001) and stored at -20°C in low TE pH 7.5. For qPCR, a multiplex protocol was implemented following Cawthon (2009). Telomere length was measured in a StepOnePlus (Applied Biosystems) or Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). For specific details regarding the telomere measurement protocol, see Section 2.3.3.

The collection of blood samples over a range of years may lead to samples being clustered during analysis by, for example, year or cohort (van Lieshout *et al.* 2020). This may lead to different conditions (i.e. laboratory conditions) among clusters (van Lieshout *et al*. 2020). To avoid blood samples being biased by such clustering, all plates used for qPCR (quantitative polymerase chain reaction) contained a random subset of samples from three different years. For a detailed description of how the analysis was completed in batches, see Section 2.3.3.

4.3.6 Storage-time effects

Blood sample storage time has been suggested to erode telomeres when stored above freezing (Tolios *et al*. 2015; but see Rosero-Bixby *et al*. 2019). However, in many studies the effects of storage on telomere measurements are unknown. We assessed storage-time effects in our samples that had been collected over a wide range of years in an experiment, where we re-extracted the DNA and remeasured telomere length on the qPCR of 80 randomly selected samples (see Section 2.3.4). We detected a negative quadratic effect of the time for which a blood sample was stored in ethanol at room temperature, where the first 5 years of storage showed a linear decline in telomere length, with a slower decline subsequently. Due to the linear decline in the first 5 years of storage, we decided to only include blood samples that were stored for five years or less in the statistical analysis. Moreover, we corrected for the blood sample storage time effect by including blood sample age (in years, as an integer) as a covariate in all models.

4.3.7 Statistical analysis

All analyses were conducted in the R environment (v. 3.5.0; R Core Team 2018), and the 'lme4' package (v. 1.1–17) was used to run the mixed models. We considered an effect to be statistically significant when *p*-values were lower than 0.05. *p*-values were calculated using the package LmerTest (version 3.1-0), using Satterthwaite's degrees of freedom.

For analysis, we only selected individuals that were cross-fostered and thus had different genetic fathers from social fathers, or genetic mothers from social mothers. Environmental effects may become stronger with age in comparison to other effects that shape a trait, as the effects of the environment may accumulate over an individual's lifetime (Price & Schluter 1991). Because environmental effects may over time erode parental age effects (Charmantier *et al*. 2006) on offspring telomere length, we selected offspring that were less than one year old for analysis.

We used within-subject centring of parental age (*sensu* van de Pol & Verhulst 2006) to separate cross-sectional (between-individual) and longitudinal (within-individual) effects. This method splits the ages of individuals into a mean age (among measurements made at different ages, reflecting between-individual effects) and a centred age (reflecting within-individual age effects). To test if the between-individual and within-individual father and mother age slopes were significantly different, we applied van de Pol & Wright's (2009) equation 3. In this model, the centred father or mother age is replaced with father or mother age, while all other variables remain the same. Age, reflecting both within- and between-individual effects, now tests for within-individual effects because the model tests each fixed effect while controlling for other effects, including mean age. The mean age now represents the difference between the mean age and centred age slopes. If the estimate of mean age in this model is close to zero and nonsignificant, the within- and between-age effects on offspring telomere length are regarded significantly different.

We tested the relationship between offspring relative telomere length and parental age using a linear mixed effect model (LMM). In this model, the offspring RTL was used as the response variable. The mean ages of the genetic fathers and genetic mother were correlated (Pearson's correlation, 0.39, t = 5.36; df = 165; p = <0.001). The mean ages of the social father and social mother were also correlated (Pearson's correlation, 0.25; t = 3.32, df = 163; p = 0.001). Therefore, we decided to run separate models for fathers and mothers.

In the father model, the following covariates were included: the mean genetic and centred father ages and the mean social and centred father ages. We included the following random intercepts: genetic father identity, social father identity and qPCR plate identity in the father model. In the mother model, the mean genetic and centred mother ages and the mean social and centred mother ages were included as covariates, while the random intercepts were genetic mother identity, social mother identity and qPCR plate identity. We initially included both the genetic brood and social brood identities, but these explained a negligible part of the variance (variance < 0.001), and so were removed.

To test for selective disappearance of father or motherss, we tested if the within-individual and between-individual slopes – in the father and mother models separately – were significantly different. In order to test if the slopes differed, we applied van de Pol & Wright's (2009) method (equation 3). In this model, the centred father or mother age is replaced with our original fixed effect (father or mother age), while all other variables remain the same in the model. Father or

mother age, reflecting both within- and between-individual effects, now tests for within-individual effects because the model tests each fixed effect, while controlling for other effects including mean father or mother age. The mean father or mother age now represents the difference between the mean age and centred age slopes. If the estimate of mean age in this model is close to zero and nonsignificant, the within- and between-age effects on RTL are considered to be alike, and it is then unlikely that selective disappearance occurs.

Birth cohorts can show differences in age-related telomere dynamics (Fairlie *et al*. 2016). Including birth cohort in our models would have provided information on how early-life experiences, such as environmental circumstances in the year of birth, can affect life-long telomere length. However, cohort is highly correlated to the age of the blood sample, and was therefore excluded from our analyses, with the consequence that we were unable to assess whether any parental age associations to offspring telomere length were affected by cohort.

We analysed a total of 166 blood samples for the father models (90 genetic fathers and 86 social fathers) and 166 blood samples for the mother models (90 genetic and 88 social mothers).

4.4 Results

4.4.1 Paternal effects

At the within-individual level, offspring RTL was negatively related to the age of the genetic father ($\beta_{\text{genetic father}}$ = -0.11, se = 0.05, t = -2.46, df = 111.6, p = 0.02; Table 4.1 and Figure 4.1). Offspring telomere length was not correlated with social father age ($\beta_{\text{social father}}$ = -0.07, se = 0.05, t = -1.26, df = 123.7, p = 0.21; Table 4.1 and Figure 4.2). At the between-individual level, an offspring's RTL was not associated with the age of the genetic father ($\beta_{\text{genetic father}}$ = 0.01, se = 0.03, t = 0.33, df = 77.8, p = 0.74; Table 4.1 and Figure 4.1). In contrast, offspring telomere length was positively correlated with mean social father age ($\beta_{\text{social father}}$ = 0.06, se = 0.03, t = 2.13, df = 89.3, p = 0.04; Table 4.1 and Figure 4.2).

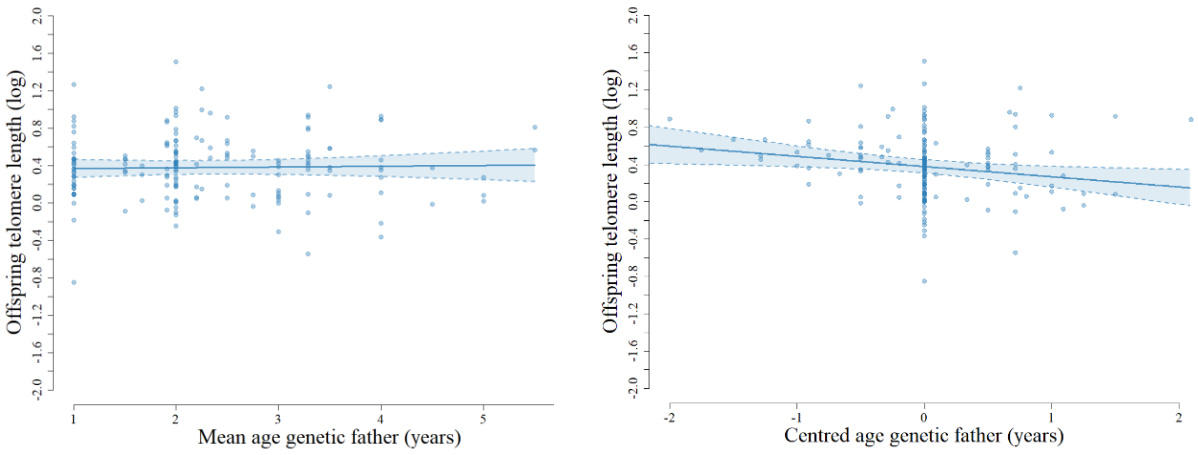


Figure 4.1. Associations of offspring RTL with mean and centred genetic father age, showing linear mixed model results. A. Between father, age of the genetic father is not associated with offspring telomere length. B. Individual genetic father produce offspring with decreased telomere length as they age. Each filled circle is one individual estimate. Thick solid lines represent model estimates and shading indicates 95% credible intervals of a linear mixed model.

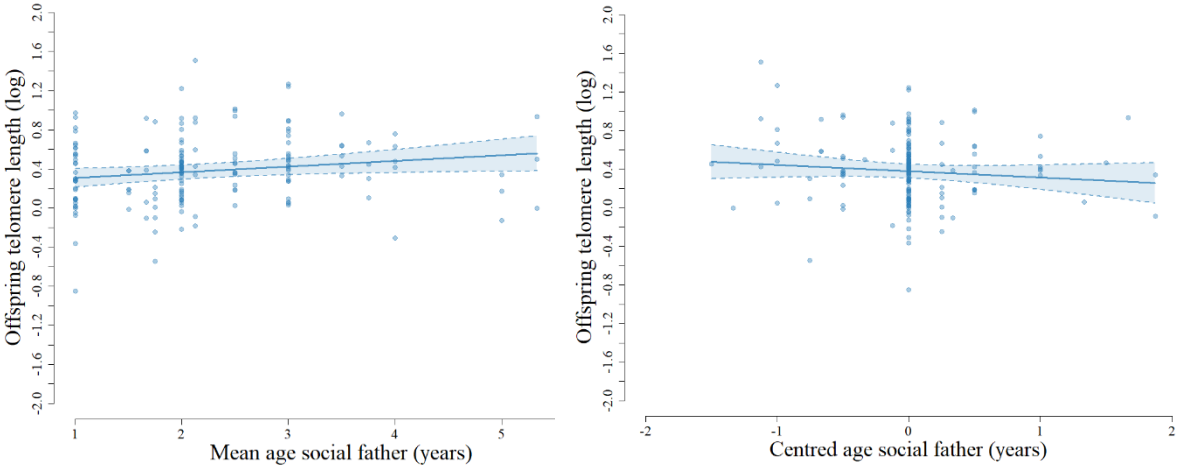


Figure 4.2. Associations of offspring RTL with mean and centred social father age, showing linear mixed model results. A. Between father, age of the social father correlates to offspring telomere length positively. B. Within Individual social fathers, age does not affect offspring telomere length. Each filled circle is one individual estimate. Thick solid lines represent model estimates and shading indicates 95% credible intervals of a linear mixed model.

The slopes showed a significant difference between the slopes of the between- and within-genetic father age effects ($\beta_{\text{different slopes genetic father}}$ = 0.12, se = 0.05, t = 2.28, df = 140.5, p = 0.02, Supporting information Table S4.1), indicating the selective disappearance of genetic fathers with offspring with smaller RTLs. The estimated difference in the slopes of the between- and within-social father ages was also significant ($\beta_{\text{different slopes social fathers}}$ = 0.12, se = 0.06, t = 2.10, df = 150.1, p = 0.04, Supporting information Table S4.1), indicating that for social fathers, selective disappearance may also occur.

4.4.2 Maternal effects

There was no association of offspring RTL with within-mother age for either genetic or social mothers ($\beta_{genetic\ mother}$ = 0.07, se = 0.05, t = 1.48, df = 139.3, p = 0.15 and $\beta_{social\ mother}$ = -0.04, se = 0.04, t = -0.94, df = 125.3, p = 0.35, respectively; Table 4.1 and Figure 4.3). Similarly, at the between-individual level, offspring RTL was not associated with the age of the genetic, nor the social mother ($\beta_{genetic\ mother}$ = 0.02, se = 0.03, t = 0.94, df = 156.9, p = 0.35 and $\beta_{social\ mother}$ = 0.05, se = 0.03, t = 1.52, df = 71.4, p = 0.13, respectively, Table 1 and Figure 4.3).

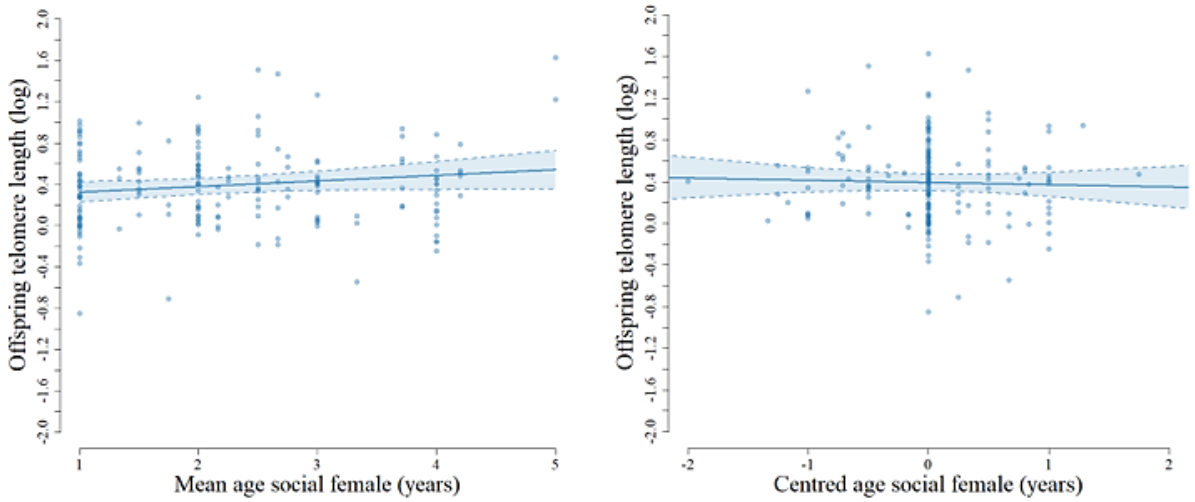


Figure 4.3. Associations of offspring RTL with mean and centred social mother age, showing linear mixed model results. A. Between social mothers, age of the social mother is not related to offspring telomere length. B. Within social mothers, offspring age is not associated with mother age. Each filled circle is one individual estimate. Thick solid lines represent model estimates and shading indicates 95% credible intervals of a linear mixed model.

Table 4.1. Effects of parental age on offspring RTL in the Lundy house sparrow population, showing linear mixed model results.

	Paternal					Maternal				
Fixed effects	**Estimate**	**se**	**t**	**df**	***p***	**Estimate**	**se**	**t**	**df**	***p***
Intercept	**0.268**	**0.100**	**2.679**	**127.0**	**0.008**	**0.246**	**0.100**	**2.512**	**126.5**	**0.013**
Between genetic parent age	0.008	0.025	0.327	77.8	0.744	0.023	0.025	0.939	156.9	0.349
Within genetic parent age	**-0.110**	**0.045**	**-2.462**	**111.6**	**0.015**	0.073	0.050	1.447	139.3	0.150
Between social parent age	**0.058**	**0.027**	**2.132**	**89.3**	**0.036**	0.046	0.030	1.521	71.4	0.133
Within social parent age	-0.065	0.052	-1.256	123.7	0.211	-0.038	0.040	-0.938	125.3	0.350
Blood sample age	-0.034	0.030	-1.132	94.1	0.260	-0.017	0.033	-0.506	105.9	0.614
Random effects	**Variance**	**Stan. dev.**				**Variance**	**Stan. dev.**			
Genetic parent identity	0.008	0.090				0.000	0.001			
Social parent identity	0.008	0.088				0.030	0.172			
qPCR plate identity	0.233	0.153				0.026	0.160			
Residual	0.079	0.282				0.071	0.266			

When comparing the slopes of genetic between- and within-mother age, the slopes were not significantly different ($\beta_{different\ slopes\ genetic\ mothers}$ = -0.05, se = 0.06, t = -0.85, df = 143.5, p = 0.40, Supporting information Table S4.1). The social between- and within-mother age slopes were also not

significantly different and $\beta_{\text{different slopes social mothers}}$ = 0.08, se = 0.05, t = 1.67, df = 150.4, *p* = 0.10, Supporting information Table S4.1).

4.5 Discussion

Our experimental cross-fostering showed that aging genetic fathers produced offspring with shorter telomere lengths. We also showed that, for genetic fathers, individuals that produce offspring with short telomeres disappear out of the population or, alternatively, their offspring disappear out of the population. Moreover, we show that offspring telomere length is positively associated with social paternal age, and that selective disappearance may also occur among social fathers that produce offspring with short telomeres. We found no association of offspring telomere length with either genetic or social mother age.

4.5.1 Genetic father and mother effects

There are several other studies that report negative genetic male age effects on offspring telomere length. Our findings add to studies in mice (*Mus musculus*, de Frutos et al. 2016), sand lizards (*Lacerta agilis,* Olsson *et al*. 2011), alpine swifts (*Apus melba;* Criscuolo et al. 2017), zebra finches (*Taeniopygia guttata,* Noguera & Metcalfe, 2018), jackdaws (*Corvus monedula*, Bauch *et al*. 2019) and Seychelles warblers (*Acrocephalus sechellensis*, Sparks *et al*. 2020).

There are three potential non-mutually exclusive explanations why older biological fathers produce offspring with shorter telomeres: 1. The gametes of older males have shorter telomeres. 2. Aging males may sire lower-quality offspring with shorter telomeres. 3. Females adjust their egg composition in response to male age, which in turn affects offspring telomere length.

The first possibility, that older males produce gametes with shorter telomeres, is a potential consequence of telomerase becoming less active or being expressed less in older males (Hamilton, 2001; Criscuolo *et al*. 2017). Less telomerase activity could lead to shorter telomeres in sperm that, when merged with the female gamete, produce lower telomere length in offspring. The mechanisms of inheritance of telomere length, and the relative contributions of the paternal and maternal genomes, including transient and possible carry-over effects during zygote development, are poorly understood (Entringer *et al*. 2018). Currently, we know that telomere length can be heritable in some species, but heritability estimates vary greatly, and may be overestimated due to the inclusion of maternal and environmental effects (Entringer *et al.* 2018). Transient effects might be expected because zygotes receive chromosomes with telomeres from both parents, which may differ in

length. Therefore, as the embryo develops different mechanisms that maintain and regulate telomere length may change and integrate the maternal and paternal telomere contribution to their soma. When telomeres in games are fully restored, or 'reset', gamete telomere length and parental telomere length will not be correlated, and the telomere effect will be transient (Liu *et al*. 2007; Entringer *et al*. 2018). However, if the maintenance and restoration mechanisms do not (completely) alter gamete telomere length, the effect can be regarded as an epigenetic inheritance effect (De Meyer *et al*. 2014), which is independent of DNA sequence variation, but dependent on a change in telomere length (Bauch *et al.* 2019). Additionally, if the maintenance and restoration mechanisms change with parental age, offspring telomere length may consequently be affected.

The second possibility is that aging sires produce offspring of lower quality (Preston *et al.* 2015). In male houbara bustards, *Chlamydotis undulata*, a decline in offspring quality (body size) with male age was theorised to occur due to a decline in the quality of male germ-line DNA, which in turn occurs through the build-up of genetic mutations due to the high replication rates in sperm (Preston *et al*. 2015). Sperm quality (DNA fragmentation, protamination, traditional semen parameters) has been linked to telomere length in previous studies in humans (Rocca *et al*. 2016; Cariati *et al*. 2016), and even though studies directly assessing links between semen quality and telomere length in non-human species are lacking at present, such a link might exist. However, in our house sparrow population a decline in the quality of sperm is less likely to occur. A previous study from Girndt *et al.* (2019) compared the sperm of old and young males, and found no significant differences in sperm quality (sperm morphology and semen volume). Even though sperm DNA fragmentation and telomere length in sperm in Girndt *et al.* 's (2019) study were not directly assessed, studies in humans indicate that there is a link between sperm morphology and DNA integrity (Tang *et al.* 2009). We therefore have no indication that DNA integrity is affected in the sperm of aging house sparrows, and it is thus unlikely that offspring are affected by sperm quality differences between old and young fathers in house sparrows.

The third explanation, which proposes that females may adjust egg composition in response to male age, has been demonstrated in previous studies (Cunningham & Russell 2000). Females may, for example, adjust egg size or testosterone levels according to male quality. Testosterone has been proposed to affect the antioxidant system in young birds, which may cause an increase in oxidative stress and, consequently, shorter telomeres (Tobler & Sandell 2009; Reichert *et al.* 2017). Additionally, females may reduce egg antioxidants when paired to older males, which in turn may also cause an increase in telomere loss (Noguera *et al.* 2018; Monaghan & Ozanne 2018). We have

not investigated the possibility of differences in the hormones or nutrition provided to the eggs of females relative to the age of their male mate, and thus cannot conclude that females respond to male age. However, males can also adjust their behaviour according to their current partner, as in our population of house sparrows, a within-father adjustment of paternal care in response to mother identity was found (Schroeder *et al.* 2016). Fathers adjusted their provisioning rates (but not their incubation efforts) when their partners showed different degrees of cuckoldry. The fathers did not change their provisioning behaviour when cross-fostered chicks were present in their broods, so fathers did not detect cuckoldry through their present offspring. A change in feeding rates in response to mother behaviour could have impacted chick growth and consequently offspring telomere length.

In addition, the timing of our experimental cross-fostering could have given genetic parents time to potentially affect offspring telomere length via incubation and other, very early life (one day) parental care effects, such as nest maintenance and brooding the chicks. Due to these incubation and very early life affects, we cannot fully attribute the effect of paternal age on offspring telomere length to a gamete effect.

Our findings contrast with studies in humans and chimpanzees, which have reported positive genetic paternal age effects on offspring telomere length (Eisenberg & Kuzawa 2018; Bouwhuis *et al.* 2018). One of the reasons, among others, why the primate studies and our study show different results could be the sperm production rate differences between mammals and birds. Humans and chimpanzees are species that have larger sperm production and produce sperm year-round. Such year-round sperm production may require more telomerase activity, or an increase in the selective loss of sperm with shorter telomeres (Froy *et al.* 2017). Species that have seasonal sperm production, such as house sparrows, are expected to have less telomerase activity in the testes, which may result in sperm having shorter telomeres and, consequently, offspring with shorter telomeres (Eisenberg 2019).

We did not detect any association between the age of the genetic mother and offspring telomere length. This is in concordance with studies that assessed the association between female age and offspring telomere length, which usually reported no effect (Froy *et al*. 2017; Bouwhuis e*t al*. 2018; Bauch *et al*. 2019; Spark *et al.* 2020). However, some studies have reported maternal age effects on offspring telomere length, such as in the great reed warbler, tree swallow (*Tachycineta bicolor*) and black-browed albatross, *Thalassarche melanophris*, where there were positive effects of maternal

age, and in the alpine swift and zebra finch, where maternal age had a negative effect on offspring telomere length (Asghar *et al.* 2015; Criscuolo *et al.* 2017; Belmaker 2016; Marasco *et al.* 2019; Dupont *et al.* 2018, respectively).

The difference in father and mother age effects on offspring telomere length may be explained by differences in the germ lines of both sexes. In mammals and birds, males produce sperm throughout their lives, while all female oocytes are formed during embryogenesis (Kimura 2008; Vizcarra *et al*. 2015; Johnson 2015). This means that telomere length of the female oocytes may be determined before birth and be less affected by female age (Aviv *et al.* 2018). However, oocytes remain in the ovary until ovulation, so eggs ovulated later in life may have undergone more oxidative stress, and consequently, telomere loss (Cimadomo *et al.* 2018). This may specifically be the case for longer-lived species, as the time period for oxidative damage to occur will be longer. In a relatively short-lived species, like the house sparrow, it is unlikely that oxidative stress in oocytes would significantly alter oocyte telomere length.

4.5.2 Social paternal effects

We found a positive association between offspring telomere length and social paternal age, but not maternal age. However, the effect size of social maternal age is similar to social paternal age, which means that a positive association between offspring telomere length and social maternal age may still exist, even though it was not statistically detected. An increase in offspring telomere length with social parental age can be the result of two, non-mutually exclusive processes: 1. Parents improve rearing conditions with age. Or 2. A quality difference between parents that produce offspring with short and long telomeres.

An improvement in rearing conditions can affect the telomere lengths of individuals because the growth period may be a critical time with respect to telomere length dynamics. During growth, telomeres shorten more quickly than during adult life due to faster cell proliferation (Zeichner *et al*. 1999; Pauliny *et al*. 2012). Telomere shortening has been linked to stress (reviewed in Reichert *et al.* 2017), and stress in the nest can lead to poor growth, which results in oxidative stress, which in turn can shorten telomeres (Hall *et al*. 2004). Parents might provide a better environment for their offspring as they age, and telomeres have been previously linked to breeding performance (Pauliny *et al.* 2006), and the quality of individuals (Le Vaillant *et al.* 2015). For example, a study on European blackbirds (*Turdus merula*) found that one-year-old birds were half as successful at finding large prey items as two-year-olds (Desrochers 1992). A link between parental age, offspring body condition

and, consequently, telomere length has been found in black-browed albatrosses (*Thalassarche melanophris*; Dupont *et al*. 2018), where it was concluded that young albatrosses raised offspring with poorer body condition (females only) and shorter telomeres.

It is possible that house sparrow parents become more adept at finding suitable prey for their offspring, becoming more efficient foragers and providing offspring with more and better nutrition, potentially resulting in longer offspring telomeres. It is also possible that parents manage to obtain higher-quality territories and nest sites as they age. House sparrow males search and inspect nest sites during winter and advertise their preferred one to females in spring (Sánchez-Tójar *et al.* 2017). Younger individuals inspect more nestboxes than older individuals. The difference in the number of inspections between old and young males is likely to be due to older individuals already being established and showing high site fidelity (Sánchez-Tójar *et al*. 2017). It is possible that younger males must settle for lower-quality nestboxes and territories, resulting in poorer nestling conditions and offspring with shorter telomeres.

However, we find no evidence that individual, aging social fathers (nor mothers) improve their offspring's telomere length. Such a within-individual increase in offspring telomere length with parental age would be expected if parents managed to obtain higher-quality territories or provided better care for their offspring as they get older. However, house sparrows show high breeding site fidelity (Sánchez-Tójar *et al.* 2017), which indicates that changes (and thus potential improvements with age) in breeding site do not occur often, potentially explaining, at least in part, why individuals do not improve their offspring telomere length with age. Instead, the lack of a within-individual, but the existence a between-individual, age-related effect in offspring telomere length might indicate a quality difference that exists early in life and persists throughout. Telomere attrition may be a cost incurred by reproduction (reviewed in Sudyka 2019) and telomere maintenance may be costly (Young 2018). Individuals with phenotypic traits that benefit them (i.e. high-quality individuals) may be more proficient in negating or bearing the negative effects of telomere maintenance (Criscuolo *et al*. 2017; Sudyka 2019). High-quality individuals may also have improved life-history traits, such as longer lifespans (Monaghan & Haussmann 2006; Bize *et al*. 2009). With improved lifespans (and thus remaining in the population at higher ages), high-quality individuals may maintain telomere length more efficiently and provide better rearing conditions to offspring throughout life, who, in turn, obtain longer telomeres.

It should be noted that separating parental social effects from genetic effects can be difficult due to relatives having a shared environment, as well as heritable genetic effects (Kruuk & Hadfield 2007). By using a cross-fostering approach we negated most confounding factors. However, cross-fostering may have its own pitfalls (Winney *et al.* 2015). We cross-fostered offspring one day after hatching, so genetic parents had some time to influence offspring with parental care before offspring were cross-fostered. This means that any pre-hatching or early-life care may have been confounded with genetic effects (Kruuk & Hadfield 2007). However, compared to the approximately two-week nest rearing, plus some post-fledging care, periods, the incubation period and the one day of parental care before cross-fostering are likely to be of low impact, and we expect that this did not affect our conclusions.

4.5.3 Selective disappearance

We detected selective disappearance in our population, at least for the genetic and social fathers. Selective disappearance hypotheses that poor-quality individuals disappear from the population, resulting in relatively more high-quality individuals at older ages (Dupont *et al.* 2018). Selective disappearance is rarely detected when studying telomeres due to the cross-sectional nature of most studies (Bauch *et al.* 2019; Eisenberg *et al.* 2019). However, one study to date, investigating parental age effects on offspring telomere length in the Seychelles warbler, found a negative within-paternal age effect on offspring telomere length, while there were no between-paternal age effects (Sparks *et al.* 2020).

We cannot discern whether selective disappearance occurs at the paternal or offspring levels. We have shown that individuals have shorter telomeres with increasing age in Chapter 3. The fathers in the present study also age, and consequently may also have shorter telomeres, which means that the fathers may disappear from the population. Alternatively, we measured the telomere lengths of fledged offspring. It is possible that older fathers are still part of the older population, producing offspring with short telomeres, which in turn died before we measured their telomere lengths. We showed previously in this same population that the eventual reproductive success of offspring was negatively affected by the father's age (Schroeder *et al.* 2015). In addition, both within-pair and extra-pair males initially increased their paternal success (total number of annual offspring sired in a year), followed by a senescent decline later in life (Hsu *et al.* 2017). If telomeres, in part, underlie, or are a biomarker of the decline in paternal success observed in Hsu *et al.* (2017)'s study, then it may be the case that selective disappearance is occurring at the offspring level.

4.5.4 Conclusion

An effect of genetic and social paternal age on offspring telomere length may have evolutionary consequences when this effect in turn affects longevity and fitness. If the paternal age effect has a negative impact on fitness, this will lead to an accelerated loss of fitness at increasing age, and thus faster senescence (Priest *et al.* 2002). Alternatively, if the paternal age effect is positive, the pace of the senescent decline will be lower at higher ages. We have previously found cross-sectional negative male and female age effects on offspring annual and lifetime fitness in our house sparrow population (Schroeder *et al*. 2015). This effect occurred before chicks were cross-fostered and was thus regarded as a genetic parent effect and it was proposed that the underlying mechanism could be telomere length (Schroeder *et al.* 2015). The findings from Schroeder *et al.* (2015) are only partially in concordance with our findings. We also detected a negative father effect on offspring telomere length, but the effect was a within-genetic father effect, while there was no association of among-genetic father age and offspring telomere length. Moreover, we did find a positive cross-sectional correlation between social father and offspring telomere length, while Schroeder *et al*. (2015) detected no social parent effect on fitness. In addition, we find no effects of neither genetic nor social mother age on offspring telomere length, while in Schroeder's *et al*. (2015) study, mother age did affect the fitness of female offspring. Due to the discrepancies, and because we made a distinction between within- and between individual parent effects, while Schroeder *et al* (2015) did not, we cannot make any inferences to telomeres being a (partial) underlying mechanism of the detected parental age effects on offspring fitness.

Supporting Information S4

Tables S4.1. Comparing slopes of paternal and maternal within and between age effects on offspring telomere length.

Effects of genetic and social paternal and maternal age, mean age and blood sample on offspring Relative Telomere Length (RTL), showing linear mixed model results. According to van de Pol & Wright (2009), the mean age now represents the difference between mean and centred age.

	Paternal				
Fixed effects	**Estimate**	**se**	**t**	**df**	***p***
Intercept	**0.268**	**0.100**	**2.679**	**127.0**	**0.008**
Between genetic parent age	**0.118**	**0.052**	**2.279**	**140.5**	**0.024**
Genetic parent age	**-0.110**	**0.045**	**-2.462**	**111.6**	**0.015**
Between social parent age	**0.123**	**0.058**	**2.104**	**150.1**	**0.037**
Social parent age	-0.065	0.052	-1.256	123.7	0.211
Blood sample age	-0.034	0.030	-1.132	94.1	0.260
Random effects	**Variance**	**Stan. dev.**			
Genetic parent identity	0.008	0.090			
Social parent identity	0.008	0.088			
qPCR plate identity	0.023	0.153			
Residual	0.079	0.282			

	Maternal				
Fixed effects	**Estimate**	**se**	**t**	**df**	***p***
Intercept	**0.247**	**0.100**	**2.512**	**116.6**	**0.013**
Between genetic parent age	-0.049	0.058	-0.848	143.5	0.398
Genetic parent age	0.072	0.050	1.444	135.1	0.151
Between social parent age	0.084	0.050	1.670	150.4	0.097
Social parent age	-0.034	0.040	-0.934	106.8	0.353
Blood sample age	-0.017	0.033	-0.508	103.1	0.613
Random effects	**Variance**	**Stan. dev.**			
Genetic parent identity	0.000	0.017			
Social parent identity	0.030	0.172			
qPCR plate identity	0.257	0.160			
Residual	0.070	0.265			

5. Low heritability of telomere length in a wild passerine

5.1 Summary

Telomeres are protective caps at the ends of chromosomes, the lengths of which may reflect the costs of environmental conditions that have been experienced by an individual and are thus considered to be a potential biomarker of senescence. Telomeres show large variation in length between individuals of the same species and age. The origin of this variation is still largely unknown, but it is important to understand the origin of variation to assess the evolutionary potential of telomere length.

We used a cross-fostering experiment, and an animal model - in which a pedigree allowed us to separate environmental from genetic effects -, to assess the genetic (heritability) and environmental contribution to the variance in telomere length of offspring, in a natural insular population of house sparrows. We found that telomere length had low heritability. In addition, neither natal and rearing broods, nor the identity of rearing fathers and mothers, explained any proportion of the phenotypic variation in telomere length. However, the year of birth explained nearly one third of the phenotypic variation. Our findings mirror other studies that have applied an animal model. The low heritability may indicate that telomere length is connected to fitness and has evolutionary potential, but that telomere length is also influenced by the environment.

5.2 Introduction

Telomeres are repetitive DNA sequences, located at the ends of the chromosomes (Meyne *et al*. 1989). Telomeres are thought to have a function in cellular replication and maintenance, and may act as a defence against cancer by triggering cell apoptosis and thus controlling uncontrolled cell proliferation (Aviv 2006). However, telomeres shorten due to the cell's imperfect replication mechanism, and stressors on the organism that cause oxidative damage, which in turn may affect telomere length (Shay & Wright 2019; Chaitelin *et al*. 2020). Maintaining telomere length may be vital to ensure the viability and chromosomal stability of cells (Blackburn 1991), especially considering that telomere length may predict individual life expectancy (Heidinger *et al*., 2012; Bize *et al*. 2009). Such a connection of telomere length to life expectancy indicates that telomere length may be a life-history marker, providing a potential proxy for future individual fitness (Cawthon *et al*. 2003), and potentially explaining variation in life-history and fitness in natural populations (Chaitelin *et al*. 2020).

It has been well documented that telomere length varies greatly between and within species (Monaghan & Haussmann 2006; Monaghan 2010). Traits with inter-individual variation are influenced by natural selection and are evolutionarily important (Reichert *et al*. 2015). Moreover, if telomere length is related to intrinsic trade-offs, phenotypes that can cope with telomere erosion may be at a selective advantage (Reichert *et al*. 2015). Therefore, the likely components of environmental variation that, in turn, affect telomere length within species has attracted attention from researchers. Most studies were conducted on a cross-sectional level, comparing individuals of different ages within a species (Pauliny *et al*. 2012) and concluded that telomere length declines with age (reviewed in Horn *et al.* 2010; overview in Dantzer & Fletcher 2015). However, such age-related conclusions must be assessed with caution because of the numerous factors that may cause between-individual variation in telomere length (Pauliny *et al*. 2012). One such factor is the heritability of telomere length.

The heritability, or specifically the narrow-sense heritability (h^2), describes the proportion of the phenotypic variance that is explained by additive genetic effects (Becker *et al*. 2015). The h^2 of telomeres has been intensively studied in eukaryotes and vertebrates, including humans, with heritability estimates varying greatly from 0 to 1 (Becker *et al*. 2015, Broer *et al*. 2013, Ashgar *et al.* 201; Dugdale & Richardson 20185). Some of the heritability estimates appear high, especially considering that telomeres may potentially be connected to fitness, as implied with the association to life expectancy, while most traits related to fitness show low heritability (Postma 2014).

There are several reasons why the estimates from several studies of the heritability of telomere length were high. First, heritability estimates are expressed in ratios, calculated as the proportion of genetic components over total phenotypic variance, which also includes other effects, such as maternal and environmental effects. As a proportion of a sum of other effects, the heritability estimate can be high when other effects are relatively low, even when the absolute additive genetic effect itself may be low (Dugdale & Richardson 2018). Not accounting for other effects that explain phenotypic variance can thus inflate heritability estimates (Becker *et al*. 2015; Dugdale & Richardson 2018).

Second, interstitial repeats that are not part of the terminal telomere genome may bias heritability estimates (Atema *et al.* 2015). This may be the case specifically for birds, where interstitial repeats appear to be numerous (Foote *et al.* 2013). qPCR estimates do not discriminate between interstitial repeats and telomeric repeats, while such a distinction may be of importance as interstitial repeats

are expected to remain unchanged in an individual's lifetime, while telomeric repeats are expected to change. However, interstitial repeats can vary between individuals (Foote *et al.* 2013) and are thus also a source of variation in telomere estimates on the qPCR. With qPCR estimates it is thus impossible to differentiate whether heritability estimates reflect variation in interstitial repeats, telomeric DNA or both (Atema *et al*. 2015).

A third reason that might potentially lead to the inflation of heritability estimates is the use of parent–offspring regressions to estimate heritability (Dugdale and Richardson 2018; van Lieshout *et al*. 2020). Parent–offspring regressions do not take multiple measurements into account and do not allow for the efficient distinction between genetic and environmental effects, because relatives will share a more similar environment than non-relatives (Dugdale & Richardson 2018). This similarity in environment needs to be partitioned out from genetic effects. Statistical, so-called animal models provide a way to separate genetic and environmental effects. An animal model uses repeated individual measurements to partition phenotypic variance into individual and residual variance, and individual variance into genetic and environmental components, allowing for the estimation of repeatability and, when adding a pedigree, heritability (Dugdale & Richardson 2018).

Another methodological issue that most studies do not control for when assessing heritability is offspring age (Reichert *et al.* 2015). Telomeres tend to change with age, and any initial genetic effect may change as environmental effects increasingly influence telomere length over time (Dugdale & Richardson 2018). Controlling for age is a necessity, as it will standardise the environmental effects that individuals were exposed to prior to sampling (Dugdale & Richardson 2018). Correcting for age does require continual data collection over a long period of time, with individuals being of known age, which may be difficult to achieve in wild study populations (Sparks *et al*. 2020).

This study aims to provide a well-founded (*i.e.* taking into account most of the aforementioned methodological issues) heritability and repeatability estimate by using an insular house sparrow population with an exceptionally extensive and comprehensive pedigree. This population is suitable for partitioning the phenotypic variation in telomere length into different components, such as genetic and environmental, by the use of an animal model due to many reasons: First, the closed nature of the population and continuous efforts to catch and band all individuals have led to almost all individuals being of known age and contributing to a single population pedigree (Schroeder *et a*l. 2012a). Secondly, many individuals are sampled multiple times, and often at similar ages in both parents and offspring, allowing for parent and offspring age standardisation. Thirdly, we cross-foster

young chicks at two days of age, which allows for the distinction between genetic and environmental effects (Winney *et al*. 2015; Schroeder *et al*. 2012b).

Aside from assessing heritability and repeatability, we then used an animal model to partition the variance in offspring telomere length into parental components, where we separate natal from rearing brood and in turn, genetic from rearing parent. In addition, we tested if the year of birth explained any of the telomeric variance found in offspring.

5.3 Methods

5.3.1 Study system

The data we used to assess heritability were collected from in 1995–2016, from a wild and insular house sparrow population located on Lundy island (51.11 N, 4.40 W), in the south-west of Britain. House sparrows are small, multi-brooded passerines (Summer-Smith 1963). The birds have a sedentary nature (Bengtson *et al*. 2004), which, combined with Lundy island being located *ca.* 20 kilometres from the coast, leads to very low immigration and emigration rates (0.5% of all recruits, Schroeder *et al*. 2015). The birds breed in nestboxes located in and around the Lundy village (Griffith *et al*. 1999, Sánchez-Tójar *et a*l. 2017).

All individual sparrows are given a unique colour ring combination and are provided with a metal numbered ring from the British Trust for Ornithology (BTO). Adults and fledglings are caught in both summer (April–September) and winter (November–March) using mist-nets or by trapping within nestboxes or funnel traps. Individuals are captured and blood sampled repeatedly throughout their lives. The ages of individual birds were calculated in years based on their cohort (year of birth). The age of each bird is known, as more than 99% of the house sparrow population were banded as nestlings or first-year juveniles. Moreover, the study has a very low catching bias and a high resighting rate of 0.96, indicating that very few birds avoid capture (Schroeder *et al*. 2015; Simons *et al*. 2015).

5.3.2 Cross-fostering experiment

Chicks were cross-fostered in all years, with the exceptions of 2008 and 2010 (Winny *et al*. 2015). Chicks were cross-fostered the day after hatching (with an exception in 2000–2003, where chicks were cross-fostered two days after hatching, Cleasby *et al.* 2010), reciprocally with any other available brood of the same age. Broods were never reduced or enlarged when cross-fostered. A proportion of broods were partially or not cross-fostered due to a lack of available broods of the same size or age, respectively, on day two. Chicks would remain in their foster-broods until fledgling.

5.3.3 Blood sample collection and telomere length measurements

We collected blood samples from juvenile (i.e. individuals that had left the nest) and adult house sparrows from Lundy Island, in the years 2003 to 2015. Blood samples were taken by puncturing the

brachial vein with a 26G needle and collecting into a non-heparinised capillary tube. The blood from each individual was stored in 1 ml of *ca* 100% ethanol at room temperature.

DNA was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Fisher) and normalised to 20–30 ng/ul. DNA was extracted using an ammonium acetate precipitation method (Nicholls *et a*l. 2000; Richardson *et al*. 2001) and stored at -20°C in low TE pH 7.5. For qPCR, a multiplex protocol was implemented following Cawthon (2009). Telomere length was measured in a StepOnePlus (Applied Biosystems) or Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). For specific details regarding the telomere measurement protocol, see Section 2.3.3.

The collection of blood samples over a range of years may lead to samples being clustered during analysis by, for example, year or cohort (van Lieshout *et al.* 2020). This may lead to different conditions (i.e. laboratory conditions) among clusters (van Lieshout *et al*. 2020). To avoid blood samples being biased by such clustering, all plates used for qPCR (quantitative polymerase chain reaction) contained a random subset of samples from three different years. For a detailed description of how the analysis was completed in batches, see Section 2.3.3.

5.3.4 Pedigree

We constructed our pedigree using the genotypes at 13 microsatellite loci to assign genetic parentage (Dawson *et al*. 2012; Schroeder *et al*. 2015). The pedigree we used contained 9,110 individuals that were sampled between 1995 and 2016. The pedigree was pruned prior to analysis to remove uninformative links (Hadfield 2010), leading to 1,105 individuals. For pedigree information, see Supporting information S5.2.

5.3.5 Statistical analysis

All analyses were conducted in the R environment (v. 3.5.0; R Core Team 2018). The T/S ratio was log transformed to fit a normal distribution (hereafter called RTL). We calculated the heritability of telomere length with an animal model (Kruuk 2004) using a Bayesian approach and the package MCMCglmm in R (version 2.28, Hadfield 2010). An animal model calculates the proportion of the phenotypic variance explained by additive genetic effects.

MCMCglmm estimates the posterior mode and 95% credible intervals (95% CI) for fixed effects and variance components, after which the heritability can be calculated. For all models (see below), the number of iterations was set to 700,000, with a thinning of 600 and a burn-in period of 15,000. We

ensured that the model converged by confirming that autocorrelation between sampled iterations was below 0.1 and using a Heidelberger & Welch (1983) test to test if samples were drawn from a stationary distribution. We used default priors for fixed effects and parameter expanded priors for the random variance structure because the random variance was small (*F* distribution: V = 1, nu = 1, alpha.mu = 0, alpha.V = 1,000).

We used a model build-up approach to assess potential confounding random effects and potential changes in heritability estimates (Charmantier *et al*. 2014), using six models. The first model contained the age of the blood sample (in years) as a fixed covariate to account for sample storage effects (see Chapter 2). The age of the individual (integer, in years) was also added as a fixed covariate. The response variable was RTL. The following random effects were added to the model: The additive genetic relationship matrix generated from individual identity linked to the genetic pedigree (animal), the identity of the bird, reflecting the permanent environmental variance explained by the identity of the individual, also accounting for common non-genetic variance due to repeated measures of the same individual (Kruuk 2004; Wilson *et al*. 2010). The qPCR plate was added to account for pseudo replication because of RTL repeated measures on the same qPCR plate.

In the second and third models, we added the brood each individual was born (natal brood) and raised in (rearing brood), respectively, to model 1. The natal brood and rearing brood effects estimated the variance among broods that can be attributed to the shared environment of individuals hatching and growing up in the same nest, over and above the variance of additive genetic effects. We did not add both the natal brood and rearing brood to the same model because not all individuals are cross-fostered, which leads to confounding effects of natal and rearing brood.

In a fourth model, we added the year of birth (cohort) to the first model to estimate the variance that can be attributed to environmental effects that occur in the year of hatching.

In a fifth model we used model four, but with a dataset that was restricted to relatively young birds, i.e. birds that were sampled in their first and second year of life. We made this selection because heritability is expressed as a ratio of total phenotypic variation (see below). As a ratio of total phenotypic variance, heritability is expected to lower as individuals age because other effects, such as the environment, start to affect individuals more strongly. This means that younger individuals should show higher heritability than older individuals (Charmantier *et al*. 2006). In order to get closer

to a heritability estimate that was less altered due to environmental effects, we selected only individuals that were sampled in their first and second year.

In the sixth and last model we added the identities of the rearing male and female as random effects to the fourth model to estimate rearing parental effects on offspring RTL.

The estimates from the MCMCglmm model were used to calculate heritability and repeatability. Narrow-sense heritability was calculated as the ratio of the additive variance component to total phenotypic variance of the trait (Wilson *et al*. 2010):

$$h^2 = \frac{V_A}{V_p}$$

Where h^2 is the heritability, V_A is the variance attributable to additive genetics and V_p is the total phenotypic variance of the trait. To calculate the total phenotypic variance, only biologically relevant variance was included. This meant that only bird identity, animal identity (as per pedigree), cohort, natal brood identity, rearing brood identity, rearing male/female identity and the residual variance were included in the calculation, depending on the model in which the effects were included.

To calculate the repeatability, the following formula was used:

$$R = \frac{V_A + V_{pe}}{V_p}$$

Where R is the repeatability and V_{pe} is the variance that can be attributed to the permanent environment of individuals.

The variance of the permanent environment of individuals, year of birth (cohort), natal early environment, rearing early environment, rearing parental male and female and the residual variance were calculated as a ratio of the total phenotypic variance.

The sample sizes used for analyses varied between models due to missing information. In total, we used 1,493 RTL measurements from 931 individuals in our analyses. When adding the rearing brood and natal brood identities, 1,096 RTL measurements from 694 individuals were used because of missing brood information. In the model with rearing males and females, we used 1,027 samples from 656 individuals. For an overview of the number of samples and individuals, see S5.1.

5.4 Results

Birds showed low repeatability for telomere length, with an estimate of 0.20 (*CI* 0.13–0.29).

The h^2 explained between 8–17% of the total phenotypic variance in offspring RTL, depending on which random factors were included in the model (Table 5.1). In the first model, the heritability was 0.17 (*CI* 0.11–0.28). This estimate excluded the qPCR plate variance in the phenotypic variance, as this variance was technical, not biological. Including the qPCR plate lowered the h^2 estimate to 0.10 (*CI* 0.05–0.16) in the first model. The permanent environment of individuals (bird identity) explained near zero of the phenotypic variation in all models (Table 5.1), which is likely due to the low number of repeated measurements within individuals.

In Model 2, where the natal brood was added, the heritability was 0.08 (*CI* 0.00–0.18). In model 3, where the rearing brood was added, the heritability was 0.08 (*CI* 0.00–0.18). In both models, the rearing and natal broods explained near zero of the total phenotypic variance (Tables 5.1 and 5.2).

In model 4, which included cohort in model one, the heritability estimate was 0.11 (*CI* 0.05–0.19). Cohort explained a greater proportion of the total phenotypic variance with an estimate of 0.27 (*CI* 0.10–0.48). When this model was used for young birds only (model 5), the heritability was 0.08 (*CI* 0.04–0.18). Cohort also explained a larger proportion of the variance when only young individuals were included (0.36, *CI* 0.24–0.72).

In model 6 we added the identity of the rearing male and female to model 4 (the cohort model). Both the rearing male and female explained near zero of the offspring's phenotypic variance (Table 5.1). The heritability in this model was also close to zero (*CI* 0.00–0.13).

For MCMCglmm model output of all models, see Table 5.1.

Table 5.1. The posterior means for the random effects in the MCMCglmm model. Below are the 95% confidence intervals shown. In all models the blood sample age and individual age were added as covariates.

Model	Animal	Bird identity	Cohort	Natal brood	Rearing brood	Rearing male	Rearing female	qPCR plate	Units	Individuals
1	**0.022** 0.012 - 0.035	**0.002** 0.000 - 0.007	—	—	—	—	—	**0.092** 0.058 - 0.130	**0.092** 0.082- 0.102	852
2 (natal brood)	**0.010** 0.000 - 0.021	**0.003** 0.000 - 0.011	—	**0.005** 0.000 - 0.014	—	—	—	**0.095** 0.062 - 0.142	**0.090** 0.077 - 0.101	634
3 (rearing brood)	**0.009** 0.000 - 0.020	**0.003** 0.000 - 0.010	—	—	**0.005** 0.000 - 0.014	—	—	**0.099** 0.062- 0.144	**0.090** 0.078- 0.101	634
4 (cohort)	**0.019** 0.008 - 0.030	**0.002** 0.000- 0.008	**0.050** 0.011 - 0.106	—	—	—	—	**0.037** 0.021 - 0.056	**0.930** 0.083 - 0.103	852
5 (only young individuals)	**0.024** 0.011 - 0.036	**0.003** 0.000 - 0.001	**0.122** 0.021- 0.266	—	—	—	—	**0.028** 0.015 - 0.043	**0.840** 0.072- 0.096	739
6 (rearing male and female)	**0.008** 0.000 - 0.019	**0.006** 0.000 - 0.017	**0.043** 0.004 - 0.101	—	—	**0.002** 0.000 - 0.007	**0.002** 0.000 - 0.006	**0.049** 0.026 - 0.078	**0.090** 0.078 - 0.103	596

Table 5.2. The calculated variance components, where the variance of additive genetic (h^2), permanent environment of individuals (V_{pe}), year of birth (V_{coh}), natal early environment (V_{nat}), rearing early environment (V_{rear}), rearing parental male (V_{male}) and female (V_{female}) and residual variance (V_r) are calculated as the proportion of the total phenotypic variance. Below are the 95% confidence intervals shown.

Model	h^2	V_{pe}	V_{coh}	V_{nat}	V_{rear}	V_{male}	V_{female}	V_r
1	**0.171** 0.109 – 0.276	**0.000** 0.000 – 0.064	—	—	—	—	—	**0.797** 0.710 – 0.873
2 (natal brood)	**0.080** 0.000 - 0.183	**0.001** 0.000 - 0.099	—	**0.001** 0.000 - 0.124	—	—	—	**0.866** 0.733 - 0.936
3 (rearing brood)	**0.081** 0.000 - 0.179	**0.001** 0.000 - 0.092	—	—	**0.001** 0.000 - 0.135	—	—	**0.864** 0.747 - 0.941
4 (cohort)	**0.114** 0.048 - 0.191	**0.000** 0.000 - 0.048	**0.266** 0.098 - 0.479	—	—	—	—	**0.588** 0.400 - 0.733
5 (only young individuals)	**0.078** 0.038- 0.180	**0.000** 0.000 - 0.049	**0.360** 0.243- 0.727	—	—	—	—	**0.447** 0.194 - 0.586
6 (rearing male and female)	**0.001** 0.000 - 0.132	**0.001** 0.000 - 0.117	**0.243** 0.056 - 0.502	—	—	**0.000** 0.000 - 0.049	**0.000** 0.000 - 0.040	**0.638** 0.407 - 0.784

5.5 Discussion

Using a mixed-model analysis of variance (animal model; Knott *et a*l. 1995; Kruuk 2004) and using a long-term longitudinal dataset, we showed that there was a small amount of additive genetic variance in telomere length in Lundy house sparrows. The permanent environment effect on telomere length was negligible in our dataset, while we did find a cohort effect. The additive genetic variance and variance explained by cohort were slightly higher in birds that were in their first year of life. The rearing brood, natal brood, rearing mother and father identities explained near zero of the proportion in phenotypic variance in offspring telomere length.

Our findings contrast with many previous studies, where telomere length heritability estimates in wild species and humans ranged from 0 to 1 (for an overview, see Dugdale & Richardson 2018, Table 1). The large range in heritability estimates may reflect interesting biological genetic variation between species, as well as methodological differences in study populations and methods (Becker *et al.* 2015). One often-proposed methodological explanation for differences in heritability estimates is the variation in the method used, such as the use of parent–offspring regressions instead of animal models (Atema *et al*. 2015; van Lieshout *et al.* 2020), especially when considering that many of the reported heritability estimates are relatively high (van Lieshout *et al*. 2020). Parent–offspring regressions do not take in account any shared environment, and thus a high covariance between parents and siblings (Kruuk *et a*l. 2004). Not accounting for or separating these non-genetic effects from genetic effects can cause an inflation of the heritability estimate (Kruuk *et al*. 2004).

We used an animal model, which partitions phenotypic variance into environmental and genetic effects (Wilson *et al*. 2010), thus accounting for a shared environment of parents and siblings, and preventing an inflation of the heritability estimate. To date, there are only a few other studies that estimated heritability by using an animal model in wild populations. Four studies that did apply an animal model in wild populations (white-throated dipper, *Cinclus cinclus*, Becker *et al*. 2015; European badger, *Meles meles*, van Lieshout *et al*. 2019; Seychelles warbler, *Acrocephalus sechellensis*, Sparks *et al.* 2020 and great mouse-eared bat, *Myotis myotis*, Foley *et al*. 2020) all found that heritability estimates were close to zero.

In contrast to the near zero or low heritabilities reported in European badgers, white-throated dippers, Seychelles warblers, great mouse-eared bat and the present study, a study from Asghar *et al*. (2015) in great reed warblers, *Acrocephalus arundinaceus,* reported a relatively high heritability estimate of 0.48. Such high heritability in the great reed warbler could be due to the use of blood samples from 8- to 10-day old chicks. As the variance that can be attributed to heritability tends to diminish with age because other factors, such as the environment, become relatively stronger, telomere length measurements in young individuals will be closer to the inherited telomere length (Charmantier *et al*. 2006). The individuals we selected as juveniles had already fledged. It is possible that nestlings express a much higher heritability, which will reflect the inherited telomere length more adequately. Whether this is the case remains a question for future research, where measuring the telomere lengths of nestlings is vital.

Some studies assessing the heritability of telomere length also find parental or nest effects. The study from Ashgar *et al.* (2015) found a strong maternal (0.47) effect on telomere length in the great reed warbler, while in the white-throated dipper there were strong nest effects (0.2, Becker *et al*. 2015). A pattern of maternal effects seems to mirror what is found in several human studies (Nordfjäll *et al.* 2010; Njajou *et al.* 2007). However, we found no maternal nor a paternal effect on offspring telomere length in any of our models, which means that mothers and fathers did not produce offspring that resembled each other more strongly than would be expected, based on their shared genes. There are several explanations that may explain the lack of parental and nest effects.

First, the lack of parental effects may be due to the occurrence of extra-pair mating (Sparks *et al.* 2020) in our population of house sparrows. Extra-pair mating would, at least for males, mean that the genetic father is not the one providing care to the offspring. However, due to our cross-fostering approach, we separated genetic parents from rearing parents and found no effects of either. Second, telomere length was measured in adults and juveniles, after the rearing period. Telomeres shorten more rapidly in early life (Hall *et al*. 2004; Salomons *et al*. 2009). As we measured telomere length in individuals that had fledged, most telomere attrition would have occurred already, potentially affected by environmental circumstances, decoupling any potential associations with parental or nest effects on telomere length. Moreover, it is likely that parental effects diminish with age because telomeres are affected by environmental circumstances (Ashgar *et al*. 2015; Sparks *et al*. 2020), as the environment an individual experiences may become more impactful in comparison to initial parental and brood effects. Third, parental and brood effects may comprise many features of the parent and brood, which may have both positive and negative effects on offspring telomere length (Becket *et a*l. 2015). The positive and negative effects can potentially cancel each other out, leading to no overall effects of parental identity and brood on offspring telomere length. Fourth, there are a low number of repeated measurements taken from offspring of individual (genetic and social) mothers and fathers, which may have made separating genetic from social parental effects impossible. Moreover, parent identity and nest identity may be strongly linked, and separating these effects requires large amounts of data (Becker *et al.* 2015).

Most studies that applied an animal model in wild populations (Becker *et al*. 2015; van Lieshout *et al.* 2019; Foley *et al*. 2020) concluded that the environment (i.e. year or cohort effects) explained a large part of the variance in offspring telomere length. Their results mirror what we found in our house sparrow population, where the variance of the year in which individual offspring hatched

explained a third of the phenotypic variance found in offspring telomere length. However, in our population, cohort is strongly linked with blood sample storage time, and we were unable to separate these two effects (see Section 3.3.6). It is possible that our cohort effects were overestimated due to blood sample storage time. Regardless, it is plausible that the cohort explains at least some of the variance in offspring telomere length. Stress is linked to telomere shortening (see overview in Reichert *et al.* 2017) and telomeres tend to shorten more quickly in suboptimal conditions; as such, the yearly fluctuations in environment can lead to differences in telomere length in both offspring and adults (Haussmann & Marchetto 2010; Young *et al.* 2013; Monaghan 2014). Differences in exposure to physiological stressors (such as food availability) during early life could lead to cohort effects, as entire cohorts will be influenced by yearly fluctuations in weather conditions and, consequently, food abundance. Indeed, it was previously shown that the mortality of house sparrows on Lundy varies widely among years, indicating a strong environmental effect (Simons *et al.* 2019). The exact cause of the variation in yearly mortality in the house sparrow is still unknown, and we can only speculate that fluctuations in environmental circumstances also affect telomere length in offspring.

To our knowledge, this is the first study that estimates heritability in a wild population that utilised a cross-fostering experiment and an animal model with a pedigree consisting of more than a thousand informative individuals. Estimating heritability of telomere length in natural populations, using individuals of known age will allow us to study the evolutionary potential of telomeres, because both natural selection and the heritability of a trait shape evolutionary responses (Kruuk 2004). Traits that are closely related to fitness tend to have low heritability as strong selection depletes variation (Mousseau & Roff, 1987; Price *et al*. 1991; Voillemot *et al.* 2012).

The low heritability of telomere length found in our house sparrow population could indicate that selection favoured individuals with long telomeres, and thus eroded some variation in a trait (Falconer & Mackay 1996), in our case in telomere length. Indications that telomere length may indeed be related to fitness are provided in Chapters 3 and 4, where we find evidence for selective disappearance of individuals with short telomeres, and disappearance of (genetic) fathers that produce offspring with short telomeres, respectively. It is thus possible that telomeres are connected to fitness, or reflect processes that are connected to fitness. Whether telomere length is linked to fitness in our house sparrow population, remains a question for the future.

Supporting information S5

Table S5.1. Number of samples and individuals per model.

Model 2 and 3	1	2	3	4	5	6	7	8	9	10-50	50-100	100-150	150-200	200-250	Total
Birds (samples)	419	136	46	19	8	5	1	0	0	0	0	0	0	0	694
Natal brood (samples)	221	101	54	41	12	10	5	4	1	3	0	0	0	0	452
Natal brood (individuals)	308	114	23	6	1	0	0	0	0	0	0	0	0	0	452
Rearing brood (samples)	216	103	53	37	14	11	6	5	1	2	0	0	0	0	448
Rearing brood (individuals)	308	114	23	6	1	0	0	0	0	0	0	0	0	0	452

Model 5 (age < 2)	1	2	3	4	5	6	7	8	9	10-50	50-100	100-150	150-200	200-250	Total
Birds (samples)	553	140	39	4	3	0	0	0	0	0	0	0	0	0	739
Cohort (samples)	0	2	0	0	0	0	0	0	0	4	1	3	1	1	12
Cohort (individuals)	0	2	0	0	0	0	0	0	0	4	4	2	0	0	12

Model 6	1	2	3	4	5	6	7	8	9	10-50	50-100	100-150	150-200	200-250	Total
Birds	394	129	45	17	7	3	1	0	0	0	0	0	0	0	596
Cohort (samples)	0	0	1	0	1	0	0	0	1	7	2	2	2	0	16
Cohort (individuals)	0	1	0	0	1	1	0	0	1	7	4	1	0	0	16
Father (samples)	56	34	26	19	10	11	5	9	5	23	0	0	0	0	198
Father (individuals)	75	39	26	21	11	8	4	5	2	7	0	0	0	0	198
Mother (samples)	63	42	28	18	6	10	7	8	3	23	0	0	0	0	208
Mother (individuals)	104	49	26	17	12	6	4	2	4	7	0	0	0	0	231

Table S5.2. Pedigree information (pruned pedigree) of the Lundy house sparrow from the years 1995 to 2016.

Relationship	n
Records	1048
Max. pedigree depth	17
Maternities	969
Paternities	974
Full sibs	1039
Maternal sibs	2577
Paternal sibs	2845
Maternal half sibs	1538
Paternal half sibs	1806
Maternal grandmothers	870
Maternal grandfathers	869
Paternal grandmothers	877
Paternal grandfathers	873
Founders	58
Mean maternal sibship size	3.27
Mean paternal sibship size	3.42
Non-zero F	726
F > 0.125	51
Mean pairwise relatedness	0.07
Pairwise relatedness > 0.125	0.20
Pairwise relatedness > 0.25	0.03
Pairwise relatedness > 0.5	0.01

6. General discussion

6.1 Research summary

In this thesis I have used, to date, one of the largest long-term datasets for a wild population to study telomere length, both methodological and biological. The use of longitudinal data has provided insights into telomere associations and life-history components that might otherwise have remained uncovered. Moreover, with the use of an experiment, I discovered that the long-term storage of blood samples is associated with relative telomere measurements on the qPCR, specifically affecting the measurements of our reference gene, *GAPDH*. Such sample storage effects may have profound implications for any study that is collecting data over a period of years. As telomeres continue to be a major focus of study, and more researchers are using longitudinal data, storage effects need to be considered when choosing a storage method. For studies that already have a large collection of blood samples, testing and consequently correcting for storage effects may be necessary. If storage effects are neglected, any biological telomere attrition may be confounded with methodological attrition. Given the long-term nature of most studies, it is important to check for any temporal effects of storage.

Once I became aware of the methodological attrition of relative telomere measures with sample storage time, I ensured I corrected for this methodological telomere attrition in further analyses. In Chapter 3, I found that telomeres shorten with age within individual birds. On the population level, I found no attrition of telomeres with age. This finding indicates the occurrence of selective disappearance, where individuals with short telomeres disappeared out of the population (van de Pol & Wright 2009). In our Lundy house sparrow population, we consider individuals to be unable to leave the study area, and due to our strong efforts to catch and observe individuals, we know when individuals have died. Selective disappearance can only be observed by obtaining both cross-sectional and longitudinal measurements in a closed population. If individuals with short telomeres are shorter lived than individuals with long telomeres, then telomeres may have fitness consequences.

Another interesting finding in Chapter 3 was that telomeres did not only appear to shorten, but some individuals also showed elongation of telomeres. I showed that this is not due to measurement error. However, as telomere length is measured as an average length across blood cells, it is also possible that the average telomere length went up due to a higher prevalence of longer telomeres. Regardless, it is still possible the telomeres elongated in at least some individuals. There are benefits

associated with telomere elongation. For example, aging has been connected to critically short telomeres, which may lead to apoptosis and senescence and thus, telomere elongation could result in a reduction in cells with critically short telomeres (van Deursen 2014). Moreover, as telomere elongation can be the result of telomerase activity, telomerase itself has been related to tissue regeneration and benefits to health parameters, at least in mice (Reichert *et al.* 2014; Simons 2015). However, elongation can also come at a cost because elongation could be energetically costly, or pose a cancer risk (Young 2018).

In Chapter 4 I was interested in the possibility that parents may pass on telomere length in a transgenerational way, so beyond the genetics and environment they provide to offspring. Using our cross-fostering experiment, which allowed me to separate genetic effects from rearing effects, I found that offspring telomere length was negatively associated with age for male biological parents. This was a within-male age effect, so individual males produced offspring with shorter telomeres as they aged. In contrast, offspring reared by older males had longer telomeres. Such an effect was only seen at the population level, meaning that individual rearing males did not increase their offspring's telomere lengths as they aged. Both the biological and social male effects that I found could be the result of selective disappearance. This selective disappearance could act at two levels: 1. Individual male level. As biological males that produce offspring with short telomeres disappear, males that produce offspring with long telomeres remain in the population at higher ages. For the social males, the males that rear offspring at higher ages with longer telomeres may also persist in the population. 2. Offspring level. Older males may persist in the population, still producing offspring with shorter telomeres, but the offspring dies before its telomeres are measured. In Chapter 3 I find selective disappearance of individuals with short telomeres. It is thus likely that the level of selective disappearance acts on the offspring level. However, whether this is truly the case is a question for future research, where the survival of both parents and offspring needs to be assessed and compared. Regardless of the level at which selective disappearance may act, the effects of paternal age on offspring telomere length can have evolutionary consequences if telomere length, in turn, affects fitness. If older fathers produce offspring with shorter telomeres that consequently (although not necessarily causatively) have lower survival and fitness, then selection is expected to favour reproduction in young individuals. As such, the population age structure could be potentially shaped by telomere length or, as causation has not yet been shown, by the underlying (physiological) process that telomere length reflects.

In Chapter 5, I was interested in the heritability of telomere length. Specifically, I was interested in partitioning phenotypic variance of offspring telomere length in a heritable and environmental component. Our study design (cross-fostering and a strong pedigree) and insular population was the perfect candidate in which to apply an animal model, which allows the partitioning of the phenotypic variance into genetic and environmental components. Using an animal model, I found low heritability of telomere length. However, cohort and thus environmental variation in the year of birth explained a large part of the variance in telomere length. This evidence for the environment affecting telomere length is in concordance with many previous studies that found cohort or year effects (Spurgin *et al*. 2018; Watson *et al.* 2015; Fairlie *et al*. 2015). The low heritability may indicate that telomere length is connected to fitness, as traits with high fitness consequences tend to have low heritability estimates (Postma 2014). However, I have not assessed associations between telomere length and fitness in our population, and thus cannot infer that telomere length has fitness consequences.

6.2 Telomeres as biomarkers of age

The idea that telomeres reflect biological age (the age reflecting life-time experiences, such as stress) has been widely proposed and studied (Mather *et al*. 2011). However, the finding that telomeres may elongate in certain individuals indicates more plasticity in telomere length than previously envisaged and indicates that telomeres may not actually reflect cumulative biological age, as originally envisaged. Telomere length may instead reflect the recent state or current quality of an individual, and also reflect the ability of an individual to maintain or even elongate its telomeres (Boonekamp *et al*. 2013). This challenges the notion that telomeres may be biomarkers of biological age, where telomeres are proposed to reflect accumulated lifetime damage. However, I show in Chapter 3 that most individuals show an overall decline in telomere length with age, and that fitting a model that allowed individuals to have varying slopes did not provide a better fit than a model that did not have varying slopes for individuals. These two findings show that, even though a few individuals showed elongation of telomeres, the general trend is still a decline, which means that telomere length, at least for most individuals, can still be a viable biomarker of biological aging.

6.3 Telomeres and causality

Simons proposed previously that telomeres might reflect an individual's state rather than play a causal role in the aging process (Simons 2015). Indeed, the evidence for a causal role in aging and senescence is weak in the current literature. Causality is usually inferred via correlation, which makes a wrong assumption (Simons 2015). However, one observation that would at least suggest

the possibility that telomeres are causally involved in aging and consequently survival is that the variance in telomere length is lower at older ages (Blasco 2005, 2007). Under this scenario the variance in telomere length reduces at older ages because, if telomere attrition causes lower survival, individuals with shorter telomeres will die sooner than individuals with long telomeres. Indeed, in Chapter 3 I show that individuals with short telomeres have lower survival in our house sparrow population, as indicated by selective disappearance. While such a finding does not confirm a causal role of telomeres in aging and survival, it does not rule out causality either.

6.4 Evolutionary potential

Individuals that can maintain telomere length (or even elongate their telomeres) may be at a selective advantage. After all, individuals with long telomeres have higher survival (as I show in Chapter 3), and pass on longer telomeres to their offspring via both genetics and transgenerational effects (as shown in Chapters 4 and 5, respectively). However, if individuals with short telomeres have lower survival, and then probably lower fitness, why has selection not favoured telomeres to elongate? Young (2018) proposes two plausible explanations. One hypothesis proposes that there are actual fitness benefits to telomere attrition. These fitness benefits would arise due to several functions that telomere attrition could have. One such function is cancer surveillance. Telomeres can act as surveyors for cancer because attrition keeps track of oxidative damage and cellular proliferation in the absence of telomerase. When cells divide without telomerase (as may occur in cancerous cells), then telomeres may become unstable, which in turn may trigger cellular responses that can lead to apoptosis (Fumagali *et al*. 2012; Campisi 2013). However, also for this hypothesis, the fitness benefits of reducing cancer risks should outweigh the costs in fitness when such reductions are not undertaken. Whether this is the case in wild populations remains a question for debate. However, even though cancer prevalence in animals is considered to be rare, the fitness consequences of cancer are still estimated to be high (Effron *et al.* 1977). This suggests that preventing cancer via telomeres could result in fitness benefits that explain why telomere attrition still occurs.

A second hypothesis proposes that telomere maintenance or elongation in itself is costly (called the costly maintenance hypothesis), under the assumption that telomere dynamics are part of the present-future trade-off and, as such, maintenance of telomeres entails resource costs. Additionally, costs could also occur due to other biological factors, for example when maintenance includes a risk to chromosomal integrity. Only when the costs of telomere maintenance outweigh the benefits, telomere attrition would be selected for. However, regardless of the pathway that would induce

costs to maintain or elongate telomere length, in the grand scheme of current-future trade-offs, it is not likely that telomeres play a major role for two reasons: First, telomere attrition can be low (for example, in humans only 20–30 base-pairs a year, Daniali *et al.* 2013). Second, maintenance can only result in small costs, along with the fact that large regions of junk DNA persist (and are thus maintained) that put the much smaller telomeric regions into the shade (Young 2018). However, telomeres could still potentially be, in some part, part of the trade-off network.

Understanding that telomere length can potentially be traded-off with, for example reproduction or maintenance will help us identify the sources of variation in life-history traits. Explaining diversity is a major goal in evolutionary biology (Stearns 1992) and it is now clear that telomeres should not be overlooked when trying to explain variation in life-history traits.

6.5 The future and telomeres

I found strong year effects when assessing the heritability of telomeres, as is shown in Chapter 5. This indicates that there is a strong environmental component to telomere length. As all individuals that were measured in this study were post-fledglings, it seems reasonable to assume that the genetic component of telomere length may have been eroded over time as environmental impacts caused telomere attrition. This means that, even though we found that telomeres were heritable, the heritability at birth may have been underestimated. In order to fully understand the heritability of telomeres, it would be beneficial to measure samples in very young birds. As we routinely take blood samples from individuals as young as two days old, the next logical step will be to measure the telomere length of these young individuals.

It will be difficult to demonstrate that telomeres play a causal role in the aging process if only correlative studies are undertaken. It is evident that experiments are required to provide insight into the causes and consequences of telomere length. Such experiments usually entail experiments that manipulate telomerase, or that increase the workload of parents or individuals via brood-size manipulations or by providing extra weight to individuals. The expectations here are that less telomerase leads to telomere loss and the stress caused by the extra workload would lead to telomere shortening and would affect survival. To date, experimental studies have mostly been undertaken in the laboratory, where telomerase knock-out studies are often used to show the potential of a causal link between telomere length and mortality (Simons 2015). However, even though telomerase deficient mice and zebrafish showed shortened lifespans, the telomere shortening was beyond that seen in natural conditions, and does thus not reflect the normal aging

process (Henriques *et al*. 2013). In order to stay within a more natural range of variation in telomere length, appropriate experiments need to be conducted in the wild. But this may be difficult, as wild populations are affected by many (environmental) conditions that, instead of the experiment, affect telomere length. Even manipulating telomerase is not straightforward, as telomerase may affect more than telomere length only (Cong & Shay 2008).

One study that did try to show causation was performed on wild great tits, *Parus major*, in the Netherlands (Atema 2017). Individuals were fitted with extra weights (up to 5% of the body mass) to increase their workloads. No effects on survival and telomere length were found. A similar experiment in Adelie penguins, *Pygoscelis adeliae,* provided similar results (Beaulieu *et al.* 2011). Whether the lack of an impact of the extra workload was due to the lack of a causal impact of workload on telomere attrition, or the impact of the extra weight being less than that of natural lifetime stresses, remains unclear. Also brood-size manipulations, where parents are expected to expend more energy on enlarged broods, which is expected to affect telomeres and consequently survival, can provide confusing results as it may be unclear what the brood-manipulation does to a parent. For example, parents may have to increase energy expenditure, which may affect telomere length and survival, as expected. Alternatively, parents may take more risks, which do not cost more energy but may yield higher provisioning rates. The laboratory and wild studies show clearly that providing evidence for a causal relationship between telomere length and mortality is not straightforward. One could also question why evidence for a causal role of telomeres bears so much weight. After all, it is possible that telomeres reflect other processes that are the actual cause of mortality. The study of telomeres is no less interesting if there is no causality of this nature because, as a biomarker of underlying processes, we can still make predictions of, or inferences to, for example, mortality. The future for telomeres thus lies in both experimenting in wild populations and trying to understand what (physiological) processes telomeres may reflect.

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