Telomeres, workload and life-history in great tits
Atema, Els

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Chapter 1

Introduction and synthesis

Els Atema
“Death takes place because a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting but finite.”

August Weismann (1881)

At the time, the means and techniques were not developed yet to test this hypothesis. But with his prediction, evolutionary biologist August Weismann turned out to be close to our current knowledge of the process of senescence. Senescence is now generally defined as: a time-related accumulation of cellular and molecular damage, leading to a decline in physiological function, and ultimately a decrease in rates of reproduction and/or survival. Although this inevitable process occurs in almost all organisms, there is considerable heterogeneity between individuals of the same species in the onset and rate of ageing. Why individuals senesce at a different pace is still not fully understood. Finding markers related to senescence is a first step in identifying factors that explain variation in the rate of senescence, which should lead to understanding the evolution of this process. The principle theme of this thesis is telomere length and dynamics, a potential marker underlying variation in life-history trade-offs and eventually senescence, and moreover a molecular trait that generally erodes over time.

**Evolutionary theory of senescence**

Although considerable insight about the process of senescence has been gained from laboratory species, such as fruit flies and mice, less is known about causes of variation in the onset and rate of senescence under natural conditions. In fact, until recently it was believed that wild animals would rarely reach an elderly stage nor show the corresponding senescent phenotype. It was thought that extrinsic factors such as predation or low food availability would cause mortality before physiological deterioration started. This was first described by Medawar (1952) who stated that wild animals “simply do not live that long”. In the last two decades evidence of senescence in wild populations is accumulating (Nussey et al. 2013), the pattern commonly found being an age-specific improvement of phenotypic traits in early-life, followed by a plateau and decline later in life. Also in the great tit, a short lived bird species, such a pattern of reproductive senescence was demonstrated, both in a British population and in the Vlieland population (Bouwhuis et al. 2009, 2010). In great tits on Vlieland reproductive success increases until the age of three years, and after that declines, likely as a consequence of senescence.

The paradox of senescence is that this process occurs in almost all species, but is seemingly running against the force of natural selection. The common basis to explain the evolution of senescence is that the force of natural selection decreases during life, because extrinsic mortality reduces survival chances to old age (Medawar 1952; Williams 1957; Hamilton 1966). Moreover, the greater part of the potential maximum
reproduction occurs during early life. Hence mutations with harmful effects later in life can accumulate, and a senescent phenotype can evolve.

Weismann was the first proposing a hypothesis explaining the evolution of ageing in the late 19th century. He suggested that organisms have to make a trade-off between investing in reproduction and maintaining somatic cells. It took a century before Kirkwood elaborated on Weismann’s hypothesis and proposed the disposable soma theory, which states that, under the assumption of limited availability of resources, individuals have to trade-off growth and reproduction against somatic maintenance (Kirkwood & Holliday 1979; Kirkwood & Rose 1991; Fig. 1). Because mortality increases with age, early-life reproduction is favoured at the expense of somatic maintenance and as a consequence senescence evolves.

![Schematic of the disposable soma theory of ageing. This assumes that the amount of resources are limited, as represented by the height of the rectangle, and therefore a trade-off should be made between somatic maintenance (light grey) and reproductive effort (or growth; dark grey).](image)

**Figure 1.** Schematic of the disposable soma theory of ageing. This assumes that the amount of resources are limited, as represented by the height of the rectangle, and therefore a trade-off should be made between somatic maintenance (light grey) and reproductive effort (or growth; dark grey).

In a wide range of species, from reptiles to birds and mammals, there was evidence for a trade-off between early-life reproduction or growth and somatic maintenance later in life, supporting the disposable soma theory (Lemaître et al. 2015). However, this energetic balance is influenced by environmental and individual quality; because resource availability and acquisition varies between individuals, variation in life-history trade-offs arises. Hence, individuals which are of good quality might be able to invest in multiple traits without apparently making a trade-off between reproductive effort and somatic maintenance (Van Noordwijk & De Jong 1986), resulting in slower senescence. Based on the disposable soma theory, molecular and cellular markers which
change with chronological age and predict biological age, could help understanding between individual variation in the rate of senescence. One might think of a wide variety of interesting biomarkers which change with age, such as oxidative stress, hormonal, immunological or DNA related biomarkers. In this thesis I used the DNA related biomarker telomere length as a read-out parameter of somatic maintenance in a free-living bird, in order to explore variation in life-history trade-offs and contribute to understanding senescence in the wild.

**Telomeres**

Telomeres are structures that generally shorten with increasing age. Measuring telomere length has received increasing interest in the field of ecology. This is because it was initially believed that the age of wild animals of unknown origin could be estimated accurately based on the length of their telomeres. This assumption turned out to be false, and currently there is interest in telomeres, because they could be used as a read-out parameter of somatic damage and the length or shortening rate correlates with experienced life stress.

*Structure and function*

Telomeres are repeats of non-coding DNA at the ends of linear chromosomes. In all vertebrates telomeres consist of the sequence 5'-TTAGGG-3' (Meyne et al. 1989). They have a function in protecting chromosome integrity and stability (De Lange et al. 1990).

Telomeres typically end in a single-stranded overhang, which together with the outer end of the double stranded telomere is folded back in the telomere strand to form a T-loop (Griffith et al. 1999; Stansel et al. 2001). This structure prevents the chromosome ends from being recognized as double stranded break. This way the T-loop keeps chromosomes from end-to-end fusion and DNA damage responses which would result in cell cycle arrest. Formation of the T-loop and the protective function of the telomeres is supported by shelterin proteins (De Lange 2005). Furthermore, telomeres act as a protective cap, providing a barrier against oxidative stress for the important, coding DNA.

*Classes of telomeres*

Three different Classes of telomeres can be distinguished, at least in birds (Delany et al. 2000). At intra-chromosomal sites interstitial telomeric repeats or Class I telomeres are located, short repeats of 0.5 – 8 kb. Interstitial telomeres are found in many vertebrates, including humans (Azzalin et al. 1997; Foote et al. 2010). They likely originate from chromosomal reorganisation events (Hastie & Allshire 1989; Lin & Yan 2008), but the function of interstitial telomeres is far from understood. We assume that they are not
susceptible to double strand breaks and unlikely to shorten with age. Hence they are not of interest as biomarkers for senescence.

At the ends of chromosomes two types of telomeres are found, distinguished by their length (Delany et al. 2000). Class II telomeres with lengths of 8 – 40 kb are involved in the protection and stabilization of chromosomes. In many species, including great tits, these telomeres were shown to shorten with age (Chapter 3). This range of the telomere distribution is of interest to ecologists and biomedical scientists as it could be used as a biomarker for biological age.

In addition Class III or ultra-long telomeres with lengths up to 2.0 Mb are found at the ends of chromosomes. They are present in several bird species (Delany et al. 2000) and also great tits have numerous ultra-long telomeres (Chapter 3). A general property of Class III telomeres is that they are extremely variable between individuals, as found in inbred mice studies (e.g. Kipling & Cooke 1990; Zijlmans et al. 1997) and in inbred chicken (Rodrique et al. 2005). The function of Class III telomeres is not understood yet, but they seem to be associated with the presence of micro-chromosomes in the genome (Delany et al. 2000). Micro-chromosomes are tiny chromosomes, typical for avian or reptile karyotypes, which despite their small size, contain about half of the genes of the genome (Smith et al. 2000; Burt 2002). A study comparing the distribution of telomeric repeats at the chromosome level in 16 bird species, found that micro-chromosomes contained a large amount of telomere sequence (Nanda et al. 2002). Similarly, Delany et al. (2000) demonstrated that Class III telomeres were more numerous in species with a higher number of micro-chromosomes. From this it was speculated that, because micro-chromosomes contain a high density of genes, ultra-long telomeres could provide an extra buffer to protect these genes from eroding (Groenen et al. 2000; Nanda et al. 2002).

Telomere shortening
Telomeres shorten with age in many species (overview in Dantzer & Fletcher 2015), including great tits (Chapter 3). With every cell cycle telomeres lose base pairs due to the end-replication problem (Olovnikov 1973): the DNA replication machinery is unable to copy the complete length of the DNA template, and as a consequence base pairs will be lost. Hence telomeres could also be seen as a buffer for important genes to not get lost during cell division.

The rate of telomere shortening is accelerated by factors related to life stress, of which oxidative stress is generally considered to be an important contributor (Von Zglinicki 2002). The balance between reactive oxygen species and the antioxidant balance plays an important role in the prediction of telomere shortening rates, at least in vitro (Von Zglinicki et al. 1995; Von Zglinicki 2002). Furthermore, oxidative stress itself can cause cellular senescence, as it triggers cell growth arrest. Also emerging evidence from in vivo studies demonstrates the relation between oxidative stress and telomere
shortening rate. For instance, in humans psychological stress results in higher levels of oxidative stress and shorter telomeres (Epel et al. 2004). In young king penguins both low survival and high growth rates were related to high oxidative stress and short telomere length (Geiger et al. 2012). Furthermore, in Adélie penguins handicapped birds increased their antioxidant capacity, whereas oxidative damage remained unchanged, a mechanism that could protect telomere lengths (Beaulieu et al. 2011).

On the other hand, telomere length can be restored by the enzyme telomerase. This enzyme carries its own RNA template to replicate telomeric sequence and restores lost telomere repeats (Blackburn et al. 1989). Although in the germ cells and stem cells this enzyme is active, in most somatic cells it is down-regulated. One explanation for this down-regulation is the relation between quickly dividing cancer cells and active telomerase (Shay & Wright 2011). But why there is such variation in telomerase activity between different types of cells is still an open question.

The balance between telomere shortening and telomere maintenance is of crucial importance for cellular senescence. A minimum number of telomeric repeats is needed for correct folding of the T-loop, hence telomere erosion affects cell fate (Stewart et al. 2003). When several telomeres are shorter than a critical length, a cell apoptosis pathway is activated and cellular senescence occurs (Harley et al. 1990). When this was discovered, telomeres became highly interesting to many researchers, as telomere length predicted cell replicative senescence. From in vitro studies, telomeres clearly play a role in cellular senescence. But are telomeres also linked to organismal senescence in multicellular organisms and healthy populations?

**Study system: Great tits on Vlieland**

Most of the work described in this thesis was done in a population of great tits (*Parus major*) on the island Vlieland, in the Dutch Wadden sea. Great tit and blue tit (*Cyanistes caeruleus*) populations have been studied on Vlieland, from 1955 up until the present day, and over this time, almost all nestlings have been ringed, parents identified and breeding success has been monitored (Van Noordwijk et al. 1981). Over 80% of the breeding individuals were ringed as nestlings and immigration and presumably emigration rates are low due to the isolated location (Van Tienderen & Van Noordwijk 1988), making the longitudinal life-history dataset very complete.

During my study (2011 – 2015) about 500 nest boxes were present in the forests and village on the island. The forest consists of 5 spatially separated patches: the biggest is the forest near the village, which is also referred to as the East, and four smaller patches which are referred to as the West. Between East and West is a dune area of 1.5 km, which the tits rarely cross, creating two subpopulations on the island (Postma & van Noordwijk 2005). In all the analyses I checked whether there were differences between these subpopulations, but this was never a significant factor in the models.
Each spring breeding activities in the nest boxes were monitored, and all nestlings and unringed parents were ringed. In winter parents were caught for a second time in the year during roost checks. We took a blood sample from adult great tits during all the catches, which were used to analyse telomere length.

**Quantifying telomere length**

Telomeres differ in length between chromosome arms, because the rate of shortening varies (Lansdorp et al. 1996), but also different Classes of telomeres were found across chromosomes. Hence a distribution of telomere lengths could be found in each sample. In general, depending on the method used for analysing telomere length, the output ranges from a point-estimate of average telomere length per sample to telomere lengths estimated per chromosome arm. In ecology there is little documentation on the suitability of the different methods for particular questions and species. Moreover, it is not well known which Classes of telomeres are best used as biomarkers for senescence and life-stress. In the first two chapters of this thesis I explored this issue for great tits, a model species often used in evolutionary and behavioural ecology.

**Terminal Restriction Fragment analysis**

The ‘gold standard’ for measuring telomere lengths is Terminal Restriction Fragment (TRF) analysis. This method involves digestion of DNA other than telomeres with restriction enzymes, pulsed field gel electrophoreses followed by in-gel hybridization of the single-stranded overhang with a $^{32}P$-labelled oligonucleotide (5'-CCCTAA-3'), and analysing images of the telomeric distribution (Haussmann & Vleek 2002; Salomons et al. 2009). Because the single-stranded overhang is labelled, only chromosome-end telomeres will be quantified, which are of interest as biomarkers. This method differs from the denaturing Southern Blot protocol, in which all telomeric repeats, including interstitial telomeres, are labelled. We preferred using the non-denaturing in gel hybridization, avoiding inclusion of interstitial repeats in the telomere estimates.

TRF analysis is time consuming and involves working with radioactivity, but a great advantage is that it results in an optical density profile of the telomere distribution in the sample. As well as calculating the average telomere length for a sample, TRF makes it possible to explore telomere dynamics for different sets of telomere lengths in the distribution. In this thesis I used a partitioning of the distribution into percentiles (10 – 90%) to analyse telomere dynamics in more detail (Chapters 3, 6). This is particularly interesting because in jackdaws (Salomons et al. 2009), common terns (Bauch et al. 2014) and humans (Kimura et al. 2007) the longer telomeres in the distribution shortened at the highest rates. We found the same patterns in great tits (Chapter 3). Because the longest telomeres are most vulnerable to oxidative damage, they lose more base pairs than shorter telomeres (Grasman et al. 2011; Verhulst et al. 2015).
Hence they are more sensitive to environmental conditions and might be more pronounced biomarkers of senescence.

**Quantitative PCR**

Quantitative PCR (qPCR) is increasingly used to measure telomeres because of the high throughput and little amounts of DNA needed. It is based on PCR reactions with primers amplifying the telomeric sequence and a primer set for an invariant control gene (Cawthon 2002, Chapter 2). By dividing the fluorescent signals of the telomeres by the invariant control gene, a ratio for the average amount of telomere per genome is obtained. An absolute measure for average telomere length per genome could be calculated by using a standard curve with known quantities of synthetic oligo (O’Callaghan et al. 2008). However, it should be noted that measurement error caused by a variety of factors is higher with qPCR than with TRF analysis (box 1). Moreover, estimates include interstitial repeats and information about the telomere distribution will be lost, which is especially a problem if a species contains high amounts of Class III telomeres which do not shorten. This is illustrated by our findings in great tits, where Class III telomeres obscured shortening of Class II telomeres when quantifying dynamics of the average telomere length of the full distribution (Chapter 3).

**Other methods**

There are other methods, often outside the field of ecology, used to quantify telomere length also (full overview in Nussey et al. 2014). More difficult to establish are quantitative in situ hybridization (Q-FISH; Lansdorp et al. 1996) and single telomere length analysis (STELA; Baird et al. 2003), but both techniques result in quantification of the telomere distribution. Moreover, the outcome of STELA is telomere length per chromosome end, which is of interest in order to discriminate between chromosomes with Class II or III telomeres. The most recent developed technique is based on Dot blot analysis, which results similarly as qPCR in a point estimate of total telomeric repeats relative to the total amount of DNA (Kimura & Aviv 2011).
Box 1. Measuring telomere length with qPCR

Quantifying telomere length with qPCR is relatively easy and analyses yield high throughput. However, there are methodological factors that influence the outcome of the qPCR analysis, which should be taken into account when validating this method. Many factors could cause variation between assays or laboratories, such as variation in DNA concentrations, composition of the master mix, choice of control gene or the qPCR thermocycler machine. Here I will discuss two factors we found contributing to variation between assays in more detail.

First, differences between extraction methods have been reported: DNA extracted from column technology resulted in shorter telomere estimates and less variance between individuals than DNA extracted by salting-out or organic extraction (Cunningham et al. 2013). Also in another study differences between methods were found; DNA extracted with spin columns yielded longer telomere estimates than protein precipitation (Tolios et al. 2015). Furthermore, DNA extracted with magnetic beads resulted in shorter telomere estimates than extraction with protein precipitation (Raschenberger et al. 2016). Passage of the membrane in the extraction columns and the vortexing during the extraction protocol affects DNA integrity.

In our own study in great tits we found that vortexing (0 – 30 secs) of extracted DNA influenced telomere length estimates, but effects were not repeatable. Furthermore, we stored a selection of great tit samples in aliquots in either glycerol buffer from which DNA was extracted with column extraction or in lysis buffer from which DNA was isolated using a protein precipitation method. qPCR telomere length estimates using DNA from both extraction methods correlated weakly (N = 40, r² = 0.29) and were longer in DNA from column extraction (3.2 ± 0.12 kb) than protein precipitation (2.8 ± 0.11 kb; F1,52.5 = 2.60; p = 0.11). Especially in long running ecological studies it is not uncommon that switches between buffers are made. Hence it is important that within experiments making use of quantifying telomere length, one storage buffer and extraction technique is used.

Second, qPCR involves temperature cycles induced by a thermocycler block. Due to inconsistent heating across the block, there are well position differences in telomere length estimates. In a systematic experiment the telomere to control gene ratio (T/S ratio) was lower close to the centre of the plate (Eisenberg et al. 2015). We also found position effects using zebra finch samples in our own laboratory. For this we used a crossed plate design: the plate was divided into quadrants and for the duplicate run the quadrants were exchanged between the diagonals. Telomere length estimates for the duplicates on different quadrants of the plate did not correlate (N = 29, r² = 0.018). This means that the outcome of telomere length varies between the different positions on the plate. Solutions would be not using the edges of the plates, use rotary qPCR thermocyclers or correct for the position effect if it is repeatable.

qPCR has the potential to be an accurate method, but estimates from gel electrophoresis have higher repeatabilities (Aviv et al. 2011). This is also illustrated by a meta-analyses on gender differences in humans: overall females have longer telomeres than males. But when repeating the analysis per molecular method, this is true for studies using gel electrophoresis, but not for qPCR (Gardner et al. 2014). Together with the above mentioned observations, qPCR did not reach the standards of accuracy that we required and thus we decided to use TRF analysis instead.
Between individual variation in telomere length

Telomere length and shortening rate highly vary between species (Haussmann et al. 2003; Dantzer & Fletcher 2015), but also between individuals of the same species. This could be due to genetic variation, environmental factors and differences in life-history strategies and interactions between these factors. A challenge handled by evolutionary biologists is disentangling the contribution of these components to trait variation. In the last three chapters of this thesis I discuss the relation between telomeres, genes and life-history.

Heritability of telomere length

Quantifying additive genetic variation of traits is of interest in evolutionary biology, because traits need to be heritable in order to evolve. In general, life-history traits have lower heritabilities than for instance morphological or physiological traits (Postma 2014). This was often interpreted as a decrease of genetic variation caused by natural selection on life-history traits. However, heritability is a ratio between additive genetic variation and total phenotypic variation, which could result in small numbers if the denominator is larger, for instance because variation caused by environmental effects is large. Therefore Houle (1992) proposed using the coefficient of genetic variation (CVA), which is given by the additive genetic variance divided by the trait mean, in order to be able to estimate evolvability. In a meta-analysis there was a positive relationship between the CVA of traits and the association with fitness, underpinning the influence of environmental factors on heritability of life-history traits (Houle 1992).

Variation in initial telomere length in the zygote, telomere shortening rate, and degree of telomere maintenance all have the potential to be genetically determined. So far mainly heritability estimates of telomere length were reported in the literature. The first heritability estimates for telomere length originated from human twin studies and parent-offspring comparisons. The strength of the heritability estimates varied between studies (Fig. 2, Chapter 4), but on average they were close to 0.7, which was also found in a large study combining several datasets resulting in a sample size of 19,713 subjects (Broer et al. 2013). Variation between studies could be attributed to differences in the relations compared, statistical approaches, and environmental effects, which are difficult to control for in human studies. Nevertheless, in humans a fair share of variation in telomere length could be explained by genetic effects. There was no difference in heritability estimates across the telomere distribution (shorter – longer telomeres) when comparing quartiles (Hjelmborg et al. 2015b). One study reported heritability of telomere dynamics during adult life, which was not as strong ($h^2 = 0.28$) as the heritability of telomere length (Hjelmborg et al. 2015b).

Results from animal studies were far less consistent (Fig.2). In sand lizards heritability was estimated to be high: 0.52 in a mother-daughter comparison or 1.23 in a
father-son comparison. In birds heritability of telomere length was on average 0.46, ranging from 0.038 in free-living white-throated dippers (Becker et al. 2015) to 0.999 in captive zebra finches (Chapter 4). However, with only seven studies estimating heritability in vertebrates other than humans there is a great need for more datasets. In particular, large studies in free-living animals could have the potential for separating genetic and environmental effects, which will contribute to a better understanding of the origin of variation in telomere length.

**Early environment and parental effects**

Low heritability estimates in animal studies could be explained by the influence of environmental effects, such as the condition in the nest of birth or year quality (Becker et al. 2015). This idea is confirmed by low a heritability estimate of 0.18 in cross-fostered collared flycatchers (Voillemot et al. 2012 but see Reichert et al. 2014). Cross-fostering is an experimental design that (partially) controls for early-environment effects. However, this seems not to be true in ad libitum laboratory conditions. In a captive zebra finch population we were unable to separate genetic and environmental effects due to low variation in environmental conditions (Chapter 4).

Besides genetic effects, in humans both maternal and paternal effects were observed. Broer et al. (2013) found that maternal effects were stronger contributors to telomere length variation than paternal effects. However, in a meta-analytic comparison which combined five human studies, no distinction in the size of maternal and paternal effects could be made (Eisenberg 2013). From animal studies no clear conclusions could be drawn yet. In sand lizards a paternal effect was found (Olsson et al. 2011b), but in four out of six bird studies maternal effects were found (Horn et al. 2011; Reichert et al. 2014a; Asghar et al. 2015a; Becker et al. 2015).

Finally, positive paternal age effects were found in several human studies (e.g. Kimura et al. 2008; Broer et al. 2013; De Meyer & Eisenberg 2014; Hjelmborg et al. 2015a). This could be explained by two (non-exclusive) alternative explanations. First, telomerase is active in the male germ stem cells (Wright et al. 1996), hence sperm telomere length increases with paternal age, which results in a positive association between paternal age and offspring telomere length. Second, germ stem cells with the shortest telomeres might selectively disappear from the stem cell population, resulting in a selected population of cells characterized by longer telomeres (Hjelmborg et al. 2015a). Only a few wild animal studies described parental age effects. In sand lizards (Olsson et al. 2011b) negative effects of paternal age were found and in reed warblers (Asghar et al. 2015a) a positive effect of maternal age was described. Obviously more studies are needed to make proper conclusions about parental effects.
Here is genetic variation at its most basic level. Teleomeres are the ends of chromosomes and are known to shorten as individuals age. This shortening is influenced by both genetic and environmental factors. In the zebra finch, for example, we observed differences in telomere length between immigrant and resident birds on the island of Vlieland. Immigrant birds born in the Eastern subpopulation had longer telomeres than resident birds born in the Western subpopulation. This could be due to higher selection pressure on telomere length in birds that migrated to Vlieland and settled in the West. We found that the length was longer in immigrant than resident birds. This could be due to higher selection pressure on telomere length in birds that migrated to Vlieland and settled in the West. We also found that resident West great tits are genetically more similar to each other than either immigrants and residents from the Eastern subpopulation or than resident birds from Vlieland. Immigrants settled in the West, and as a consequence, the grey circle among the bird studies depicts our heritability estimates (0-1). The grey area reflects the expected range of heritability estimates (0-1). The grey circle among the bird studies depicts our heritability estimate in the zebra finch.

**Figure 2.** Overview of estimated heritability of telomere length in human studies (both twin and parent-offspring comparison), sand lizards (parent-offspring comparison) and birds (sibling-, parent-offspring comparison and 'animal model'). The grey area reflects the expected range of heritability estimates (0-1). The grey circle among the bird studies depicts our heritability estimate in the zebra finch.
Box 2. Factors underlying variation in telomere length in great tits

We intended to explore genetic and environmental factors underlying variation in telomere length in great tits on Vlieland (N = 123 individuals; 289 samples). There is genetic differentiation in micro-satellites between the two subpopulations (East and West) on the island (Postma et al. 2009). Immigrants settle mostly in the West, and as a consequence immigrants and Vlieland-West great tits are genetically more similar to each other than either of them to Vlieland-East great tits. Also, there is a consistent genetic difference in clutch size between the two areas on the island (Postma & van Noordwijk 2005). Our telomere phenotype dataset was small and includes too few direct relatives to calculate heritability. Therefore, as a rough estimate of genetic effects on telomeres we compared telomere length of first samples corrected for age between the subpopulations and immigrants and residents. We found no differences in telomere shortening rate in any of the comparisons (not shown).

There was a trend that telomeres were longer in great tits born in the Eastern subpopulation than in West (difference: 751.44 ± 404.24 bp; F1,94 = 3.46, p = 0.067). There was no difference in telomere length between resident and immigrant birds (difference: 244.79 ± 255.56 bp; F1,114 = 0.92, p = 0.35) and also no differences between the genetic clusters based on micro-satellites, that is East-Vlieland versus West-Vlieland and immigrants (difference: 81.90 ± 235.71 bp; F1,109.2 = 0.12, p = 0.73). Note that the individuals in our dataset were not genotyped.

Finally, we investigated environmental effects on telomere length later in life by distinguishing between East and West as areas of breeding. Previously, production of recruits and adult survival were shown to be consistently higher for birds breeding in the East than in West (Postma & van Noordwijk 2005). Telomere length did not differ between the Eastern and Western subpopulations (difference: 239.57 ± 278.43 bp; F1,113 = 0.74, p = 0.39). However, there was an interaction between breeding area and migrant status (F1,114 = 6.22, p = 0.014, Fig. 3). In the West, the area with lowest fitness perspective for great tits, telomere length was longer in immigrant than resident birds. This could be due to higher selection pressure on telomere length in birds that migrated to Vlieland and settled in the West. We can only speculate that resident West-Vlieland great tits were able to cope with more difficult environmental conditions given their shorter telomeres. An alternative explanation is that the immigrant birds in the East were Vlieland birds born in natural cavities.

Altogether, telomere length is longer in resident East great tits than in resident West great tits. Currently we are unable to disentangle the genetic and environmental effect. An interesting continuation would be estimating heritability of telomere length in nestlings with an ‘animal’ model, a technique that allows for separation of genetic and environmental factors.

Figure 3. Telomere length plotted for the breeding subpopulations and migrant status (res = resident, immi = immigrant). Plotted values are model estimates corrected for age and gel differences (random effect) ± standard error. Numbers in the graph indicate the number of males. Different letters indicate significant differences according to statistical results.
Telomeres and life-history

In vitro experiments demonstrated that telomeres are causally involved in cellular senescence. But how do telomeres relate to senescence and fitness in multicellular organisms? And what are the effects of environmental factors on telomere length and dynamics? My main interest in this thesis was telomere length, and especially telomere dynamics, as a marker of cellular senescence, in order to understand the trade-off between reproductive effort, telomeres, and ultimately survival.

Reproductive success

Under the assumption that resources are limited, a trade-off between reproductive effort and somatic maintenance could be expected, according to the disposable soma theory (Kirkwood & Holliday 1979; Kirkwood & Rose 1991). This association could be investigated both in cross-sectional datasets comparing telomere length and reproductive success in the same year, or in longitudinal datasets by investigating telomere shortening following reproduction. In general, longitudinal studies yield higher power to detect differences between individuals, because they can be correct for selective disappearance of poor quality individuals, which might be underestimated effects in cross-sectional datasets (Van de Pol & Verhulst 2006). Moreover, longitudinal analyses yield additional information to cross-sectional analyses, and with longitudinal analyses it could be tested whether an association with telomere length could be explained by telomere shortening rate independent of telomere length.

There were several cross-sectional studies in wild animals testing for the association between telomere length and reproductive success (Fig. 5). In dunlins (Pauliny et al. 2006), king penguins (Le Vaillant et al. 2015) and sand lizards (Olsson et al. 2011a) small or no associations between the two traits were found. In leatherback turtles more successful individuals had longer telomeres (Plot et al. 2012), whereas in male common terns more successful individuals had shorter telomeres (Bauch et al. 2013). In great tits we found no association between telomere length and reproductive success (Chapter 6). Although most estimates were positive, the findings are mixed (Fig. 5), demonstrating the complexity of this relationship. The association between telomere length and reproductive success in common terns was quadratic, in the sense that the most successful individuals lost fewer telomere base pairs than the intermediate successful birds (Bauch et al. 2013). This suggests there is high heterogeneity between individuals due to the interaction between differences in individual quality and reproductive effort.
Figure 4. Two alternative scenarios for the relationship between telomere shortening and reproductive success. In the left panels, solid lines represent telomere dynamics with increasing age in reproductive successful individuals, and dashed lines in unsuccessful individuals. We assume that telomere shortening rate reflects the effort put into reproduction. The right panels are the outcomes of reproductive success given the effort made during reproduction. (a) Individuals that have no reproductive success lose more telomeres than successful individuals. Unsuccessful individuals put high effort into reproduction, but given their state, are both unable to gain reproductive success and maintain telomere length. Hence, their high effort results in low success. (b) Individuals which have reproductive success lose more telomeres than unsuccessful individuals. Successful individuals put high effort in reproduction, but this comes at the cost of losing telomeres. Nevertheless, their high effort results in high success.

Two alternative scenarios could be expected from longitudinal datasets. In the first scenario (Fig. 4a) individuals without reproductive success (-) lose more base pairs than individuals with reproductive success (+). Despite their high effort, identified from telomere loss, reproductive success is lower in these individuals. In the second scenario (Fig. 4b) individuals with reproductive success lose more telomere base pairs than individuals without reproductive success. In this scenario there is a trade-off between reproduction and somatic maintenance. In the literature there is evidence in wild animals for both scenarios (Fig. 5). Individuals with the highest reproductive success
lost less telomere base pairs in humans (Barha et al. 2016) and great tits (Chapter 6), in agreement with scenario (a). In common terns individuals with the highest reproductive success lost most telomeric sequence, consistent with their findings in the cross-sectional dataset, and supporting scenario (b) (Bauch et al. 2013). But in king penguins there was no correlation between reproduction and telomere dynamics (Le Vaillant et al. 2015). Both in common terns and great tits the association between reproduction and telomere shortening was most pronounced in the longest telomeres in the sample telomere distribution.

It is difficult to interpret these results based on four studies in different species. Next to variation in life-history strategies and telomere dynamics, there is also variation caused by for instance differences between methods to quantify telomere length (qPCR versus gel electrophoresis). It is important to keep in mind that the great tit dataset contained only males; hence it is good to note that the correlation we find is mainly due to territory defence and chick rearing activities. In female birds the costs of reproduction might be different, as they produce eggs and, at least in great tits, incubate the eggs. Costs of reproduction might be even higher in female mammals, due to changes in physiology and body composition during pregnancy and lactation.

*Experimental approaches to disentangle trade-offs*

In addition to studying the effects of natural variation in life-history traits, experimental approaches are helpful in understanding causality. It is well-established that trade-offs have to be made between reproduction and survival, this has been demonstrated in experiments where brood size was manipulated. Costs could be explained by several mechanisms, but likely contributors were immune functioning and increased oxidative stress. Also telomere shortening was a candidate to be affected by experimentally increased reproductive effort (Fig. 5). When reproductive effort was pushed upwards by increasing brood size in a laboratory setting in zebra finches, increased brood sizes negatively affected telomere erosion (Reichert et al. 2014b). Not only brood size, but also reproduction itself impacted telomere shortening in zebra finches, although these effects did not last in the long-term (Heidinger et al. 2012). Also in the wild, trade-offs with telomere maintenance had to be made in blue tits when brood sizes were increased (Sudyka et al. 2014).

However, manipulating energy expenditure by handicapping birds with a backpack did not influence telomere dynamics (Beaulieu et al. 2011, Chapter 6). In our manipulation male great tits had to carry 5% of their body mass during one year, which seemed a substantial handicap (Chapter 5). We found small effects of the additional mass on the state of the males; for instance body mass increased and males were less likely to occupy a nest box for roosting in winter. But, we found no long-term fitness consequences in terms of reproductive success or survival. And also telomere dynamics
were not affected by the manipulation of workload. Apparently great tits were able to cope with this load, which was comparable to transmitters used in small passerines.

![Diagram of telomere distribution](image)

**Figure 5.** Overview of studies on the association between telomeres and reproduction. We divided the studies in (i) cross-sectional designs, comparing telomere length and reproduction at the same time, (ii) longitudinal designs, comparing reproduction and subsequent telomere dynamics and (iii) studies manipulating reproductive effort (brood size manipulation in zebra finch and blue tit, handicap with backpack in Adélie penguin and great tit). Note that telomere length in dunlins was quantified with the program Telometric, which produces biased estimates. See text for references.

**Survival**

In humans, telomere length was affected by lifestyle factors that were also related to lifespan. For instance smoking, physical inactivity and diet were all to a certain extent related to telomere length (e.g. Aviv et al. 2009; Freitas-Simoes et al. 2015; Mundstock et al. 2015). Moreover, humans with longer telomeres had a higher chance of survival, in a meta-analysis (Boonekamp et al. 2013). Also in evolutionary ecology there is
accumulating evidence for a positive association between telomeres and survival (Fig. 6). Although generally in literature positive correlations between telomere length and survival were found, there are several exceptions. In cross-sectional studies a non-significant positive relation was found in dunlins (Pauliny et al. 2006), soay sheep (Fairlie et al. 2015) and barn swallows (Caprioli et al. 2013) and a negative correlation in water pythons (Ujvari & Madsen 2009). It is good to note that, unexpectedly, in water pythons telomeres were reported to elongate with age. In longitudinal studies in barnacle geese (Pauliny, Larsson & Blomqvist 2012), blue tits (Sudyka et al. 2014) and soay sheep (Fairlie et al. 2015) non-significant positive correlations were found. In great tits individuals with longer telomeres had a higher chance of survival, but this did not reach statistical significance (Chapter 6).

A few studies demonstrated early life telomere length was a better predictor for survival and lifespan than telomere length later in life. The first evidence came from a captive zebra finch population, in which individuals with the longest telomeres as nestling, had the longest lifespan (Heidinger et al. 2012). This positive association between telomere length and lifespan became weaker with increasing sampling age. These findings were confirmed by a positive correlation between early life telomere length and lifespan in wild reed warblers (Asghar et al. 2015b). Finally, in jackdaws there was no association between survival and nestling telomere length in a cross-sectional dataset, but in the longitudinal dataset nestling losing fewer base pairs had higher recruitment chances, suggesting telomere shortening rate is a better predictor for survival (Boonekamp et al. 2014).

In common terns the association between telomere length and survival was most pronounced in the longer telomeres within individuals (Bauch et al. 2014). This is in agreement with the shortening rate with age being highest in the longer telomeres in jackdaws (Salomons et al. 2009), common terns (Bauch et al. 2014) and great tits (Chapter 3). These findings seem paradoxical, given that in vitro the shortest telomeres trigger cell growth arrest when they are below a certain threshold (Harley et al. 1990). These in vitro studies suggested causal involvement of telomeres in the process of senescence, but this has so far not been supported by any in vivo studies. In fact, in a recent review of telomerase knockout and overexpression studies little support was found that telomeres cause senescence (Simons 2015). Instead telomere length could be interpreted as a proxy for individual quality or read-out parameter of experienced life-stress, as also found in our study in great tits (Chapter 6).
Telomeres shorten with age and are related to biological age or senescence. Evidence for the relevance of telomeres as biomarkers for individual quality or life-stress is accumulating. In this thesis I was interested in telomeres in great tits, a short-lived passerine bird. My first aim was to establish accurate methods for quantifying telomere length in great tits. In chapter 2 my co-authors and I describe the development of an invariant copy number control gene, the key step for developing usage of qPCR in a new species. We developed primers on a sequence in the great tit GAPDH gene, which cross-
amplified in blue tits. For future studies the importance of a proper control gene and thorough validation of the qPCR technique should not be neglected. In current studies the appropriate details about validation steps are often lacking (see MIQE guidelines; Bustin et al. 2009). The high throughput of qPCR is an enormous advantage, but the lower repeatability and point estimate of the average telomere length made the method less suitable for measuring telomere length in great tits. Hence we analysed telomere length with TRF analysis, resulting in information regarding the full telomere distribution.

In chapter 3 my co-authors and I show that shortening of Class II telomeres was masked by Class III telomeres in great tits. This made qPCR an inappropriate method to quantify telomere dynamics in great tits. With the TRF method we showed that Class II telomeres shortened with age, but Class III telomeres did not. Moreover, in the distribution of Class II telomeres the longer telomeres lost base pairs at the highest rate. These long repeats were more vulnerable to damage, likely to be caused by oxidative stress. Based on these findings it is highly recommended to explore the telomere distribution with TRF analysis when studying telomeres in new species. When Class III telomeres do not obscure shortening of Class II telomeres, qPCR could be used as a quicker method to estimate telomere length.

My second goal was to identify factors underlying variation in telomere length. In chapter 4 my co-authors and I show that the heritability of telomere length in captive zebra finches was extremely high. This dataset did not allow for separation of genetic and environmental effects. Because environmental variation was small, we concluded that this likely overestimated our heritability estimate. Furthermore, in preliminary analyses in great tits it was suggested that next to genetic background, environmental effects are important determinants for telomere length. More studies disentangling genetic, parental and environmental effects in wild animals are needed for better understanding of the interaction of factors underlying variation in telomere length, mainly in early life as this seems the best predictor for lifespan.

Third, in chapter 6 I was interested in the correlation between telomere length and dynamics and life-history traits. My co-authors and I found no association between reproduction and telomere length in cross-sectional analyses. However, in longitudinal datasets, individuals with higher reproductive success in terms of recruits lost fewest telomere base pairs. We argued that telomere dynamics could be used as proxy for individual or environmental quality. Although our estimates were in the expected positive direction, there was no association between telomere length or dynamics and survival. Since great tits are short-lived birds, stochasticity in extrinsic mortality factors, which do not relate to telomere shortening, might play an important role. Due to practical reasons our study was limited to male great tits. It would be interesting to include females in future analyses. Other costs of reproduction could be expected in this
sex, since female great tits are responsible for nest building, egg production and incubation.

Finally, in addition to the study of genetic components and effects of natural variation in life-history strategies, we studied the effects of prolonged higher workload of male great tits to establish whether there was a causal relationship between life stress and the rate of senescence and telomere shortening in chapter 5 and 6. We found short-term effects on male state, but no long-term fitness consequences of our treatment. Moreover, there were no effects of the additional mass on telomere dynamics, fitting with the lack of long-term fitness consequences. This means that despite the stressor of the additional mass during one year, great tits were able to cope with this and similar equipment, such as transmitters.

To conclude, Class II telomeres shortened with age and were positively but weakly correlated with fitness components in great tits. Telomere shortening in this species was masked by the abundance of Class III telomeres, which did not erode. What the function of these ultra-long telomeres is remains an open question. Nevertheless, with this thesis another step in gaining insight in telomeres as a marker for underlying variation in phenotypic quality and life-history trade-offs was made.
Chapter 1

Quantifying telomere length
PART I

Quantifying telomere length
Chapter 2

GAPDH as control gene to estimate genome copy number in greens, with cross-amplification in blues.

Els Atema
Kees van Oers
Simon Verhulst

Ardea, 101, 49–54 (2013)